Open-BIO
Opening bio-based markets via standards, labelling and procurement

Work package 5
In situ biodegradation

Deliverable N° 5.7

Marine degradation test lab assessment: Marine degradation test of bio-based materials at laboratory and mesocosm scale assessed

Public summary

Version: 1

Novara, September 2016

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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. One part of the project (WP5: In situ biodegradation) deals with research on the biodegradation behaviour of bio-based polymers in natural environments: soil, freshwater and the marine environment.

The biodegradation of materials is still difficult to predict in the marine environment. The ability to biodegrade can vary a lot and depends on the properties of the materials and on the (local) environmental conditions of the marine ecosystem. Bio-based polymers are not biodegradable per se and biodegradation needs to be assessed for each product. A lot of the work currently carried out within Open-Bio, is dedicated to get more insight in how to deal with biodegradability issues of bio-based polymers in different environmental settings. A solid testing scheme for the biodegradation of plastics in the marine environment does not exist so far. There are considerably less test methods available in the literature for marine than for freshwater or soil environments and further investigations are needed to explain the differences observed between the various marine habitats.

Currently, few test methods for the assessment of the biodegradation of materials in the marine environment are available from ISO (International Organisation for Standardisation) and ASTM (American Society for Testing and Materials). No European CEN (European Committee for Standardisation) test method has been developed so far. The available test methods concern the biodegradation under aerobic conditions. One test method only addresses disintegration and it is not suited to measure biodegradation (ASTM D7473-12). The only standard specification defining requirements concerning disintegration, biodegradation and environmental impacts in marine conditions (ASTM D7081-05 in combination with test method ASTM D6691-09), has been withdrawn and is currently under revision. This standard specification was targeting biodegradability in aerobic seawater. Early 2015, the Belgian private non-profit agency, Vinçotte, introduced the certification scheme for the “OK biodegradation MARINE label” based on the criteria of ASTM D7081-05.

So far, biodegradation test methods for polymers in the marine environment are very specific (only natural seawater was considered) and poorly standardised. As compared to: freshwater, soil and compost conditions, the marine environment, especially seawater, is considered less aggressive from a biodegradation point of view. A major difference between marine environment and soil is the biofouling (colonization by micro and macreoorganisms) effect on the biodegradation process which has not been studied in depth in the marine environment and its effects remain practically unknown.

In order to better understand the great variation within the entire marine ecosystem, a set of well-defined marine habitats needed to be identified and characterised according to their physical, chemical and biotic properties. This information should provide the baseline for

conditions as natural as possible to be applied for each habitat-specific standardised test. The conditions and the possible modifications needed to obtain relevant test schemes are reviewed in deliverable 5.5[1] produced within the framework of the Open-Bio project. As a result within the Open-Bio project new testing methods are currently being developed for the biodegradation in the sandy eulittoral (intertidal beach) zone, in the sublittoral (benthic) zone at the water/seafloor interface and in the pelagic (free water column) zone.

In nature there are several more sets of conditions that are important: many marine areas are very low in oxygen (hypoxic) or free from oxygen (anoxic), vast regions are covered with very fine sediment (mud) and are cold. Notwithstanding the high diversity of different marine habitats, the study of all of them is out of the scope of the project. The main goal of this deliverable (5.7) was to develop new foreground knowledge by defining new testing schemes based on the adaptation of existing test methods for relevant environments in order to be able to present the new laboratory, mesocosm and field test results and compare them comprehensively. A secondary aim was to improve the new developed laboratory test methods based on inter-laboratory tests and on the results of parallel field and mesocosm tests in order to obtain better and more robust testing schemes for the three marine habitats (eulittoral, benthic and pelagic) considered in the project.

Part 1:

The overall goal of this task was to develop laboratory methodologies and dedicated testing schemes for marine biodegradation of plastics under three distinct marine environments. To reach this, four test materials: LDPE (Low Density Polyethylene; negative control), PBSe (PolyButylene Sebacate), PBSeT (PolyButylene Sebacate co butylenTerephtalate) and PHB (Polyhydroxyalkanoate Copolymer; positive control) were tested at laboratory scale, following three different newly developed test methods (under evaluation). The biodegradation in benthic (interface sandy sediment/seawater) and eulittoral condition (intertidal beach sandy sediment) was measured according to two new methods proposed in the project, while the biodegradation in pelagic condition (free seawater) was determined based on a modified version of ASTM D6691 (lower nutrient content). Five laboratories carried out the biodegradation tests measuring the CO_2 production or the O_2 consumption using seawater and sediment coming from Salamis Island (Greece) and Elba Island (Italy). The tests were repeated for two consecutive years.

During the first year of testing some problems were identified and improvements were implemented following their evaluation. The proposed test methods were proven suitable to measure the rate of biodegradation of plastics in the three different marine conditions but optimization of the test parameters (e.g. amount and shape of test item, amount of inoculum, addition of nutrients, etc) is required to shorten test times and improve the reproducibility.

In general, from the test materials point of view, PHB was biodegraded in all conditions. It is therefore considered a good positive control. On the contrary, as expected,

[1] Deliverable 5.5: Review of current methods and standards relevant to marine degradation. Down-loadable from www.biobasedeconomy.eu

LDPE remained completely intact up to the end of the test. The polyesters PBSe and PBSeT showed a steadily increasing biodegradation with the time of their exposure to eulittoral and benthic conditions while inhomogeneous trends were observed under pelagic conditions.

Concerning the inocula: the benthic sediment, in general, showed a high organic content indicating high microbial activity, leading to clearly distinguishable biodegradation but also a tendency of an overproduction of CO_2 especially during the first year. Furthermore, this high biological activity in the case of a readily biodegradable material as PHB combined with the low diffusivity of air in the water led to a formation of anaerobic zones on the surface of the sediment (in the lab). The eulittoral sediment, having low organic content produced generally reliable results even if the biodegradation rate was rather low. Finally the free seawater (pelagic) showed reliable results for PHB but not for the polyesters. The reason of this is probably the very low concentration of microorganisms in this environment.

Concerning the test method point of view it appeared that the benthic method was characterized by not clear and unequivocal results due to CO_2 overproduction. Progress was made during these two years to improve the benthic test method, but the test methodology still needs further improvement. Further research is advised, following the suggestions reported in this deliverable (such as administering test items in powder form, adding additional nutrients to the test medium, increasing the test item concentration, refining the sediment pre-treatment phase, etc.). Regarding the eulittoral method fewer modifications are needed. The main problem observed was attributed to the low biodegradation rate. The use of powdered test samples in conjunction with additional nutrients appeared to result in an increase of the biodegradation rate, but this also needs to be further investigated. The pelagic test method also seems be promising as similar biodegradation trends were obtained in both years. Only the biodegradation results obtained with aliphatic aromatic copolyesters were not reproducible, probably due to the low microorganism concentration. Further research is needed to improve the pelagic test method.

Part 2:

The goal of the second part of this task was to develop a stand-alone mesocosm test to assess the degradation of polymers under partially controlled marine conditions. A closed-circuit tank system which mimicked the same three shallow-water habitats as in the laboratory tests, namely eulittoral (intertidal beach scenario), pelagic (water column scenario) and benthic (sublittoral seafloor scenario, sediment-water interface) within a single system was developed. The mesocosms were placed in a climate chamber where light, temperature, water movement, tides and water quality could be controlled complementary to laboratory tests. The volume of several hundred litres per habitat, and the use of natural sediment and seawater provided experimental conditions that were closer to the natural environment, and thus also allowed a link to field tests. Three independent mesocosm tank systems were run in parallel, two times consecutively, for the duration of one year each. The same polymers as in the laboratory tests (PHB, LDPE, PBSeT and PBSe) were tested.

The developed mesocosm system was well suited for the intended tests. Its simple construction and low technical effort proved to be reliable and efficient. Generally, all tested polymers, except the negative control LDPE, showed disintegration, with a differentiation by

habitat and polymer type. However, there was a high variability in the rate of disintegration between replicates and between the experiments in year 1 and 2. Part of this heterogeneity could be explained by inhomogeneities in e.g. water movement, illumination and fouling, or slight differences in the system between the years, e.g. sediment grain size. Another part of the heterogeneity could not be explained *ad hoc*, and also be attributed to natural variations of the matrices water and sediment, and the microbial community therein. The observation of this high variability in a partially controlled test system and the analyses of the possible causes provided important information for the validation of laboratory and field tests.

The biodegradation of polymers, defined as the remineralisation to CO_2 (and/or CH_4) and water, and the conversion to biomass can only be directly measured in closed test systems where either CO_2 development or O_2 consumption is monitored, but not in the open tanks of the mesocosms. Therefore, material disintegration of polymer samples was estimated by the determination of lost area % over time. This technique provided a simple method to assess disintegration of polymer films, but had some intrinsic inaccuracies. The method was based on the visible lack of material and thus could only produce results once advanced disintegration had led to the perforation of the film. To assess the polymer degradation independently from eventual fragmentation there were also analytical methods applied to assess polymer disintegration on a macromolecular level like GPC and MALDI-TOF, but the results obtained did not show their suitability.

Methods that determined the mechanical properties of the tested materials at different exposure times gave satisfying results in case of slow degradation, but could no longer be applied for samples at an advanced stage of disintegration. Linked to the reliable determination of degradation is another question that could not be sufficiently addressed. Up to now no method could be applied that allowed to directly link the polymer biodegradation and specimen disintegration in the lab test to the disintegration of the same polymer in the mesocosm tests. Such a methodological link would be useful for a calibration of the tests in the laboratory and in mesocosms, and furthermore also for field tests, and should be developed in further projects.

One disadvantage of the mesocosm tests performed was the relatively slow disintegration achieved at the applied temperature of 21 °C, which extended the necessary experiment duration to up to 1 year. Slight modifications of the conditions within a natural range, e.g. higher temperatures or the addition of nutrients could accelerate the disintegration and render the tests more practical.

The outcome of this part of the project is the proposal of a mesocosm test system suited to be applied independently from direct access to the sea with relatively low technical and financial effort. The mesocosm system can fill the gap of knowledge on the performance of biodegradable polymers under environmentally relevant marine conditions, in three of the most important coastal habitats. It can be developed into an additional test method to link the series of laboratory tests to field tests in the sea.

This ensemble of tests will open the possibility for materials and products to be tested under marine conditions in a reproducible environmentally relevant manner, and help society, producers and policy makers to verify claims of biodegradability.

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2 Content of the Deliverable and methodology used

The Open-Bio WP5 aims to develop testing methods and specifications on marine biodegradation of bio-based materials. The development of new test method means to prepare, to carry out and to validate it. The strategy adopted in the project was to simulate the biodegradation in marine conditions at three levels: laboratory, mesocosm and field. The laboratory scale correspond to the simulation in very low scale with very stringent control of the parameters of the reaction and the possibility to measure the mineralization of the bio-based polymers, that is the organic carbon of the test material transformed in CO_2 by microorganisms. Clearly the laboratory test is an “accelerated” test performed under optimal temperature and nutrient conditions. To validate the biodegradation laboratory tests, in parallel also pilot tests were carried out exposing the polymer samples directly to real-life conditions (seawater and sediment) on two different scales: mesocosm-scale, i.e. larger scale tests carried out in the laboratory, and field tests, that meant that samples were left the marine ecosystem under study for several months up to a year. The goal of this deliverable 5.7 is the assessment of the laboratory tests on bio-based plastic materials. This report describes the marine environments reproduced, the test materials, the apparatus and the instruments used in laboratory and the biodegradation and disintegration results.

3 Introduction

This document is based on the outcome of Deliverable 5.5 and on the different experiences of the partners. The goal of this deliverable is to develop a methodology and testing scheme for marine biodegradation and to validate it with tests in laboratory scale. As described in the Deliverable 5.5, within the last few decades, plastics have revolutionized our daily lives, but unfortunately this wide production and use of plastic items has had a negative impact on the environment. Since the 1920s, research was concentrated on the production of polymers with the goal to produce light plastic material, easy to transform and model, but with an high resistance to the degradation, similar to metal products or other (natural) material. These studies between 1950 and 1960 resulted in the development of very good materials with high chemical, thermic and light resistance to satisfy a lot of applications, substituting metals or wood and simplifying our life. This development caused an increase in plastic consumption and an accumulation of these in the environment. Globally, we use in excess of 260 million tonnes of plastic per year, accounting for approximately 8 per cent of world oil production(1). In the last years there was a significant development of biodegradable plastics and also biodegradable bio-based plastic. The double objective is clear: to manage the environmental impact and to reduce the use of fossil resources to produce these materials. The biodegradable plastics are designed to biodegrade in specific environments: composting, soil, water. These items must be collected in controlled manner (i.e. separate domestic organic waste collection) and treated in composting plants. In the agricultural field there are important applications such as in mulching film or string and clips that after use ultimately end up in the soil where they should be degraded due to the microbial activity. Unfortunately, overall in these last years, a wide amount of plastic: items, e.g., shopping bags, fisher gear, are littered in the environment without control. Soil and sea are “full” of plastic that as a result of mechanical and physical weathering may fragment and result in the formation of so called microplastics. Especially, the sea environment is particularly affected by this problem. A lot of scientific publications and reports from environmental non-governmental associations showed the dangerous effect on the animals: birds and turtles died for plastic ingestion. Furthermore, evidence is emerging that plastics with environmental contaminants can transport these compounds to organisms at various trophic levels(2). Cleary, the only solution to confront this big and global problem is to avoid the uncontrolled dispersion of plastic, but to avoid dispersion completely seems unrealistic,. It is possible to reduce the problem with a solid environmental education, especially with the young generation, but it is necessary to put in the field actions and ideas to mitigate the littering problems. One of these it is the possible development of biodegradable plastic that can be biodegraded and transformed in CO_2, water and new biomass if left on the beach or in open sea in relatively short time without the generation of microplastics. Obviously this isn’t the solution, but it is a possibility to decrease the environmental risk. Thinking about a possible strategy to face the problem, the biodegradable plastics can be a useful tool but is necessary to study the biodegradability using the robust and validated test methods.

3.1 Marine ecosystems

Deliverable 5.5 described the marine habitats where the plastics have been found. Soil, waste water treatment plants, or the composting process are nowadays (as a result of several decades of study) more easy to simulate at laboratory scale and also in pilot scale than marine conditional testing which is still in its infancy. The marine ecosystem is made by a set of rather different habitats: solid, liquid or interface solid/liquid system, aerobic or anaerobic conditions[3]. To reproduce all different environments, summarized in Table 1, was not possible in the Open-Bio project, so it was decided to concentrate on three different habitats: Eulittoral, Benthic and Pelagic.

Table 1. List of marine habitats and habitats effectively studied in Open-Bio project

Habitat	Condition	Habitat reproduced in Open-Bio project
Supralittoral zone	Aerobic, partially buried in the dry sediment or in soil	✗
Eulittoral zone	Aerobic, partially buried in sediment/sand with regular humidification by tidal water	✓
Pelagic zone	Aerobic, open ocean water, free floating	✓
Benthic zone (Sublittoral)	Aerobic, lying on the bottom	✓
Deep sea	Aerobic, lying on the bottom	✗
Buried in the sediment (sublittoral or deep sea)	Anaerobic, buried in the sediment	✗

The ecosystems were chosen for different reasons: the deep sea is very interesting with a high accumulation of plastic debris[4] [5], but in this first phase it was decided to test the biodegradability of plastics in habitats more easy to reproduce in laboratory. Also was preferred to focus the effort on aerobic biodegradation leaving out the anaerobic (sediment) habitat. About the supralittoral zone it was considered, in this preliminary phase, the biodegradation of plastic in soil (studied in WP5). Based on these considerations the preparation of the experiments started with the goal to reproduce: plastics brought to the shoreline by the tides or the storms (Eulittoral), plastics floating in open sea (Pelagic) and plastic that reach the bottom (Benthic/Sublittoral).

4 Material and methods

4.1 Seawater and sediment

4.1.1 Eulittoral zone

The sediments were collected in two different locations: Isola d'Elba (Italy) by Open-Bio partner Hydra and Salamina Island (Greece) by Open-Bio partner AUA. The samples of sediments were withdrawn from the eulittoral zone of the shoreline, where the tides maintain wet (sandy) sediment. The two samples had rather different features: the Elba sediment is similar to a sandy sand, while the sediment from Salamina Island is more coarse with stones. The samples were stored in plastic containers, directly transferred to the partners involved in biodegradation laboratory tests, and stored at 4˚C until further use in the tests. The biodegradation of plastic was tested after burial of the test item in the sediment. Figure 1 gives an image of the received sediment of Greece, while Figure 2 shows the sediment of Italy. The sandy sediment of Greece contained more impurities (mainly sea shells and big stones) compared to the sediment from Italy. These objects were removed from the sandy sediment. The eliminated parts from the sediment of Greece are shown in Figure 3. Before the start-up, the excess water of both sediments has been eliminated by filtering using a coarse filter paper or a fine grid.

Figure 1. Visual presentation of the sediment of Greece (before screening)

Figure 2. Visual presentation of the sediment of Italy (before screening)

Figure 3. Visual presentation of the removed particles (mainly sea shells and big stones) of the sediment of Greece

4.1.2 Benthic zone (Interface sediment/seawater)

The seawater and sediment were collected in two different locations: Isola d'Elba (Italy) by Open-Bio partner Hydra and Salamina Island (Greece) by Open-Bio partner AUA. The samples were collected respectively at a depth of 40 m and 30 m, stored in plastic containers, directly transferred to the partners involved in biodegradation laboratory tests, and stored at 4˚C until further use in the tests. The biodegradation of plastic was tested by placing a test item on the sediment surface at the interface sediment/seawater. Plant material, sea shell, pieces of driftwood, or other large pieces of material were removed from the sandy sediment. Before the start set-up, the seawater and the sediment were separated by filtering using a coarse filter paper or a fine grid.

4.1.3 Pelagic zone (free seawater)

To reproduce in laboratory scale this habitat, seawater was collected in two different locations: Isola d'Elba (Italy) by Open-Bio partner Hydra and Salamina Island (Greece) by Open-Bio partner AUA. An uniform sample of seawater was collected at a depth of 15-20 m , stored in plastic containers, directly transferred to the partners involved in biodegradation laboratory tests, and stored at 4˚C until further use in the tests. The biodegradation of plastic was tested after milling and dispersing the test materials in seawater. There was no need to remove plant material, sea shell, pieces of driftwood, or other large pieces of material because of the purity of the two seawaters.

4.2 Test materials and sediments: Chemical and physical characterisation

In Table 2 are reported the plastic materials used to validate the biodegradation tests in the different marine habitats. The low density poly-ethylene (LDPE) represents the negative control.

In Table 3 (Greek sediments) and Table 4 (Italian sediments) the characteristics of inoculum; seawater and sediments used are reported. The data summarized in Table 3 and Table 4 are measured by the different partners and refer to the first year test

Table 2. List of test samples and their relative chemical characterization

Test material	Note	TOC (%)	TC* (%)	H* (%)	N* (%)
Low Density Polyethylene **LDPE** (negative control)	Grade: LUPOLEN 2420K Lyondelbasell Film 30 microns	85.03	85.37	14.68	< 0.1
Polybutylene Sebacate **PBSe**	Aliphatic polyester Film 25 microns	65.26	65.58	7.69	< 0.1
Polybutylene Sebacate-co-butylenterephtalate **PBSeT**	Aliphatic-Aromatic polyester Film 25 microns	65.25	65.81	9.54	< 0.1

Polyhydroxyalkanoate Copolymer **PHB** (positive control)	Grade: Mirel™ P5001 It is a compound > 70% PHB copolymer, plasticizer, fillers Film 90-100 microns	47.82	49.11	6.03	0.52

*: Calculated by elementary analysis

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Table 3. Sediments and seawater collected in Salamina Island (Greece)

Parameters	Benthic				Pelagic				Eulittoral			
	Nov	AUA	OWS	LeAF	Nov	AUA	OWS	LeAF	Nov	AUA	OWS	LeAF
TS (%)	21.1	26.2	27.7	n.d.	n.d.	n.d.	n.d.	n.d.	86.1	77.1	93.7	n.d.
Water content* (%)	78.9	73.81	72.3	n.d.	n.d.	n.d.	n.d.	n.d.	13.95	22.87	6.3	n.d.
VS (% on TS)	3.6	n.d.	4.2	n.d.	n.d.	n.d.	n.d.	n.d.	1.20	n.d.	1.5	n.d.
pH	8.69	7.78	8.20	n.d.	8.06	7.98	7.80	n.d.	8.53	7.85	8.80	n.d.
EC ($\mu S\ cm^{-1}$)	n.d.	n.d.	n.d.	n.d.	n.d.	54339	n.d.	n.d.	n.d.	n.d.	1350	n.d.
Total N (mg kg^{-1} TS)	n.d.	735	1187	n.d.	n.d.	n.d.	219	n.d.	n.d.	170	324	n.d.
Total Organic C (% wt/dry wt) [b]	n.d.	0.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.075	n.d.	n.d.

Table 4. Sediments and seawater collected in Elba Island (Italy)

Parameters	Benthic				Pelagic				Eulittoral			
	Nov	AUA	OWS	LeAF	Nov	AUA	OWS	LeAF	Nov	AUA	OWS	LeAF
TS (%)	59.6	n.d.	64.3	65.5	n.d.	n.d.	n.d.	4.2	79.2	n.d.	86.4	81.1
Water Content (%)	40.4	n.d.	35.7	34.5	n.d.	n.d.	n.d.	95.8	20.3	n.d.	13.6	18.9
VS (% on TS)	4.61	n.d.	4.8	5.6	n.d.	n.d.	n.d.	15.7	0.45	n.d.	0.4	0.57
pH	8.68	n.d.	8.4	7.49[a]	8.07	n.d.	7.9	8.3	8.55	n.d.	7.9	8.0
EC ($\mu S\ cm^{-2}$)	n.d.	n.d.	4580	n.d.	n.d.	n.d.	53500	n.d.	n.d.	n.d.	3560	n.d.
Total N (mg kg^{-1} TS)	n.d.	n.d.	1086	308	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1475	7.4
Total C (g kg^{-1} TS)	n.d.	n.d.	n.d.	113	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.1

[a] determined in the bottles after mixing of sediment and seawater after 52 days of storage

4.3 Biodegradation tests: Laboratory scale

The biodegradation in laboratory was measured in seawater/sediment interface (Benthic), sandy sediment (Eulittoral) and in free seawater (Pelagic). For the first two environments the protocol analysis was prepared and sent to the laboratory involved (refer to **Annex 1** and in **Annex 2** for complete description of the two protocols). The determination of the biodegradation in free seawater was carried out following the ASTM D6691 - 09

"*Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Seawater Inoculum".* In this test the seawaters (from Elba Island and Salamina island) were used directly without any treatment. Only KH_2PO_4 (0.1 g/L) and NH_4Cl (0.05 g/L) were added to the seawaters. They serve as nutrients for the micro-organisms, which will biodegrade the specimens. It was decided to add 0.05 g NH_4Cl per liter seawater instead 0.5 g/L NH_4Cl (as prescribed in ASTM D6691) because it was assumed that a high concentration of NH_4Cl would produce an very low C/N ratio and could lead to nitrification. In a typical case 250 ml of seawater and 60 mg of test material are used. Assuming the total absence of nitrogen in the seawater, the addition of 0.5 g/L NH_4Cl results in a nitrogen content of about 33 mg in each bottle, which is relatively high compared to 60 mg of sample (corresponding to about 30/35 mg of TOC). The reactors are stored at 20-28˚C with magnetic stirring. The biodegradation results in transformation of the organic carbon in the plastic test items to CO_2 and new biomass. The biodegradation is determined measuring the CO_2 developed or the oxygen consumed.

In Figure 4, Figure 5 and Figure 6 examples of apparatus used for the determination of biodegradation respectively: in seawater/sediment interface (simulation of Benthic zone), in wet sandy sediment (simulation of Eulittoral zone) and finally in free seawater (simulation of the pelagic zone) are shown.

Figure 4. Set-up biodegradation in seawater/sediment interface. Simulation of Benthic zone.

Figure 5. Set-up biodegradation in sandy sediment. Simulation of Eulittoral zone.

Figure 6. Set-up biodegradation in free water. Simulation of Pelagic zone.

Table 5 (Eulittoral zone), Table 6 (Benthic zone) and Table 7 (Pelagic zone) summarize the experimental set-up adopted by the laboratories partner of the project that performed the marine biodegradation tests.

Table 5. Experimental set-up biodegradation in laboratory Eulittoral zone simulation

Parameters	Novamont	AUA	OWS	LeAF	BASF
Reactor volume (L)	3.0	4.0	4.0	2.0	0.25
Type	Static (closed vessel)	Static (closed vessel)	Static (closed vessel)	Static	Static (closed vessel)
Temperature (°C)	28 ± 1	25-28	28 ± 1	20 ± 1	25 ± 1
Sample characteristics	Singular piece of plastic film	Piece of plastic film	2 pieces (LDPE, PBSe, PBSeT) + 1 piece (PHA)	PHB: single piece (2.1x4.0 cm) Other: 2 pieces (4.0x4.0 cm)	Singular piece of plastic film
Quantity of sample (mg)	Around 100	1000 mg C of sample	100	Around 100	20 mg C of sample
Quantity of inoculum (g)	Around 400	Around 350	400	400	75
Measurement method	CO_2 titration	CO_2 titration	CO_2 titration	CO_2 titration	Pressure loss / O_2 consumption
Chemical reagent	KOH 0.5 M and HCl 0.3 M	KOH 1M and HCl 0.25M	KOH 0.5 M and HCl 0.1 M	KOH 0.5 M and HCl 0.3 M	$NaOH/Ca(OH)_2$
Nutrients	/	0.1g N/1g C [a]	/	/	after 129 d: add. 6-10 mL artificial seawater with 0,05 g/L NH_4Cl and 0,1 g/L $KH_2(PO_4)$

[a] In the form of nitric nitrogen $N\text{-}NO_3$

Table 6. Experimental set-up biodegradation in laboratory Benthic zone simulation

Parameters	Novamont	AUA	OWS	LeAF	BASF
Reactor volume (L)	0.250	4.0	0.250	0.250	0.250
Type	Static (closed vessel)	Static (closed vessel)	Static (closed vessel)	Static (closed vessel)	Static (closed vessel)
Temperature (°C)	28 ± 1	25-28	28 ± 1	20 ± 1	25 ± 1
Sample characteristics	Singular piece of plastic film	Piece of plastic film	Singular piece of plastic film	Singular piece of plastic film	Singular piece of plastic film
Quantity of sample (mg)	Around 20	0.08 – 0.2 - 0.2 and 0.5 g C of sample	20	25-35	20 mg C of sample
Quantity of inoculum (sediment) (g)	Around 30	Around 170	30	Around 30	30
Measurement method	CO_2 titration	CO_2 titration	CO_2 titration	CO_2 titration	Pressure loss / O_2 consumption
Chemical reagent	KOH 0.5 M and HCl 0.3 M	KOH 1 M and HCl 0.25 M	KOH 0.5 M and HCl 0.1 M	KOH 0.5 M and HCl 0.3 M	$NaOH/Ca(OH)_2$
Seawater (ml)	Synthetic (70)	Natural (around 400)	Natural (70)	70	Natural 70
Nutrients	/	0.1g N/1 g C [a]	/	/	/

[a] In the form of nitric nitrogen $N\text{-}NO_3$

Table 7. Experimental set-up biodegradation in laboratory Pelagic zone simulation

Parameters	Novamont	AUA	OWS	BASF
Reactor volume (L)	0.250 – 0.500	0.250	0.500	0.25 (0.304 total volume)
Type	Static with stirring	Static with stirring	Static with stirring	Static (closed vessel)
Temperature (°C)	28 ± 1	25-28	28 ± 1	25 ± 1
Sample characteristics	Plastic powder	Plastic specimen	Milled plastic film	Plastic film
Quantity of sample (mg)	Around 20 - 30	Around 20-25	60 mg	20 mg C of sample
Quantity of inoculum (ml)	82 - 123	82-85	250	100 g
Measurement method	BOD manometric oxygen consumption (OXITOP) plus CO2 titration	BOD manometric oxygen consumption (OXITOP) plus CO_2 titration	CO_2 titration	Pressure loss / O_2 consumption
Chemical reagent	KOH 0.5 M and HCl 0.3 M	KOH 1 M and HCl 0.25 M	NaOH pellet and HCl 0.1 M	$NaOH/Ca(OH)_2$
Nutrients	KH_2PO_4 (0.1 g l^{-1}) NH_4Cl (0.05 g l^{-1})	F2 fertilizer (0.131 g l^{-1}) [a]	KH_2PO_4 (0.1 g l^{-1}) NH_4Cl (0.05 g l^{-1})	KH_2PO_4 (0.1 g/ L) NH_4Cl (0.05 g/L)
Testing method	ASTM D6691 - 09	ASTM D6691-01	ASTM D6691 - 09	ASTM D6691 - 09

[a] In F2 Nitrogen is in form of $N\text{-}NO_3$

4.4 Disintegration tests: Mesocosm test

The mesocosm represents the connection between the laboratory and the real environment (Figure 7). It is a sort of pilot-scale that could be very useful to characterize the materials, especially the determination of the disintegration.

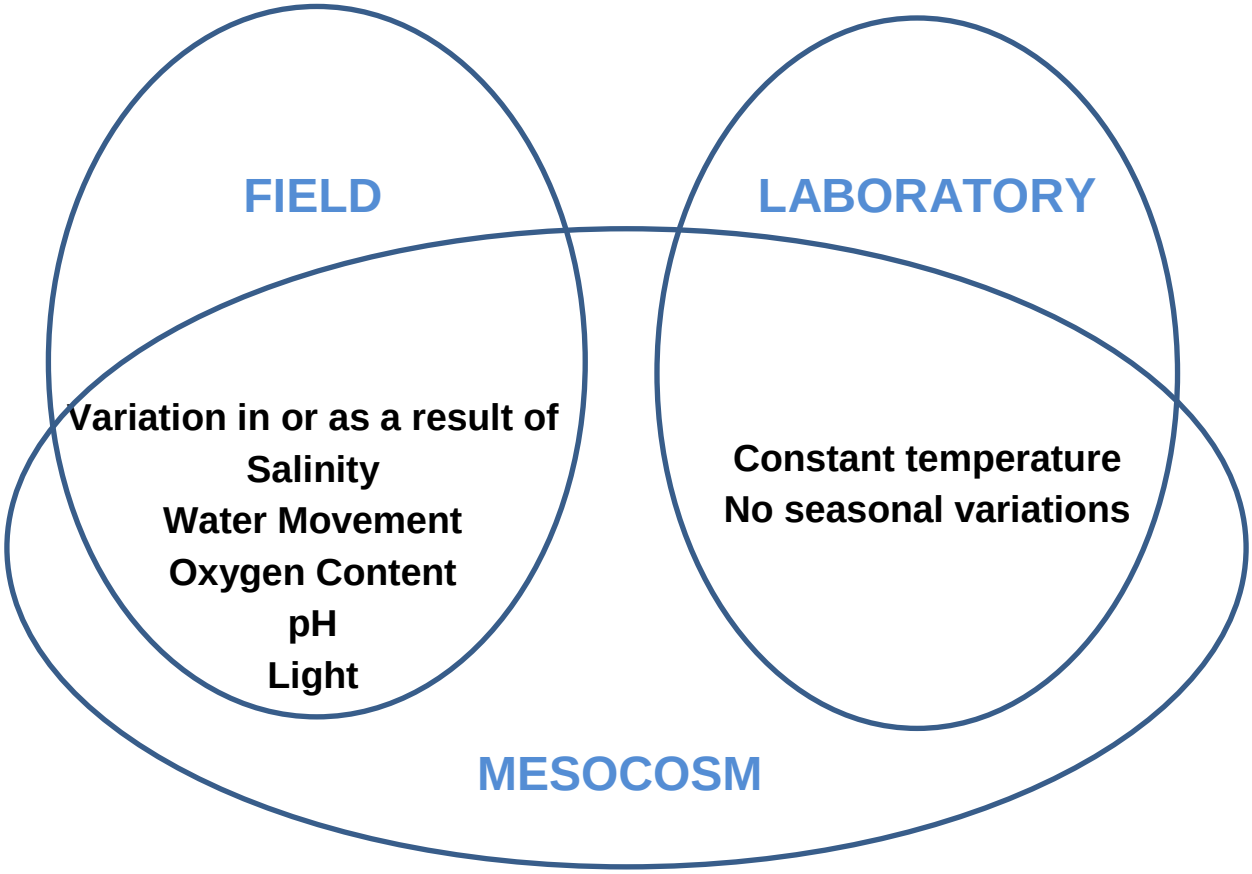


Figure 7. Mesocosm connection activity between the field and the laboratory scale approach.

Test materials PBSeT, PBSe, PHB and LDPE were placed into frames (PE-HD 300) and covered with 4 x 4 mm diamond-shaped mesh (LDPE, General Cable), then are exposed to the three different environments, in three HDPE plastic tanks (Dolav GmbH, Bad Salzuflen) with inner dimensions 93 x 113 x 60 cm. The three tanks are filled with seawater, seawater/sediment and sandy sediment coming from Elba island (Italy). At different times, the samples are collected and cleaned and following parameters are determined: mechanical characteristics (measured in order to follow their degradation), photo documentation, mass loss (where possible), GPC and Maldi TOF analysis (only for the second year samples). During the exposition the abiotic parameters are checked and kept stable. In this way the seasonal variations are excluded in the laboratory. The disintegration of the tested polymers was measured by determining the area of the remaining polymer. Detailed results about mesocosm degradation of polymers where reported and described into the Deliverable 5.7 Part 2 where the validation of laboratory and mesocosm results is performed.

5 Laboratory results of year 1

The results obtained during the first year of experiments from the different laboratories are summarised in this chapter. Results are firstly discussed per laboratory and finally all results per “environment” are taken together to better understand the trends and the differences.

5.1 Laboratory: Novamont

5.1.1 Biodegradation in Eulittoral zone

The tests were performed according to Annex A and Table 5. The preliminary phase, carried out in order to verify the endogenous respiration in the different reactors and also to oxidize the eventual ready biodegradable organic matter present, was protracted for one week. Samples of: LDPE, PBSe and PBSeT were cut into about 5.5 cm × 5.5 cm pieces. One piece of these materials corresponds to approximately 100 mg. Test material PHB was cut into smaller pieces, because the thickness was higher. The same procedure was used both for the Greek sediment that for Italian sediment. In total, 12 reactors were prepared. Two reactors for each specimen (4 specimens), 2 control reactors, 2 cellulose paper * 2 sediments. The period of time between the carbon dioxide analysis by means of titrations was variable. The first titration was performed after seven days in both tests, then because of the low biodegradability of the test items and low background activity of the sediment, titrations were performed every two weeks. In Figure 8 and Figure 9 the biodegradation curves of Eulittoral environment (Greek and Italian sediment respectively) are given. The tests lasted respectively 368 and 329 days. The biodegradation processes were still in progress, only the PHB in the test with the Italian sediment reached the plateau phase. In Table 8 and Table 9 the final results are summarized.

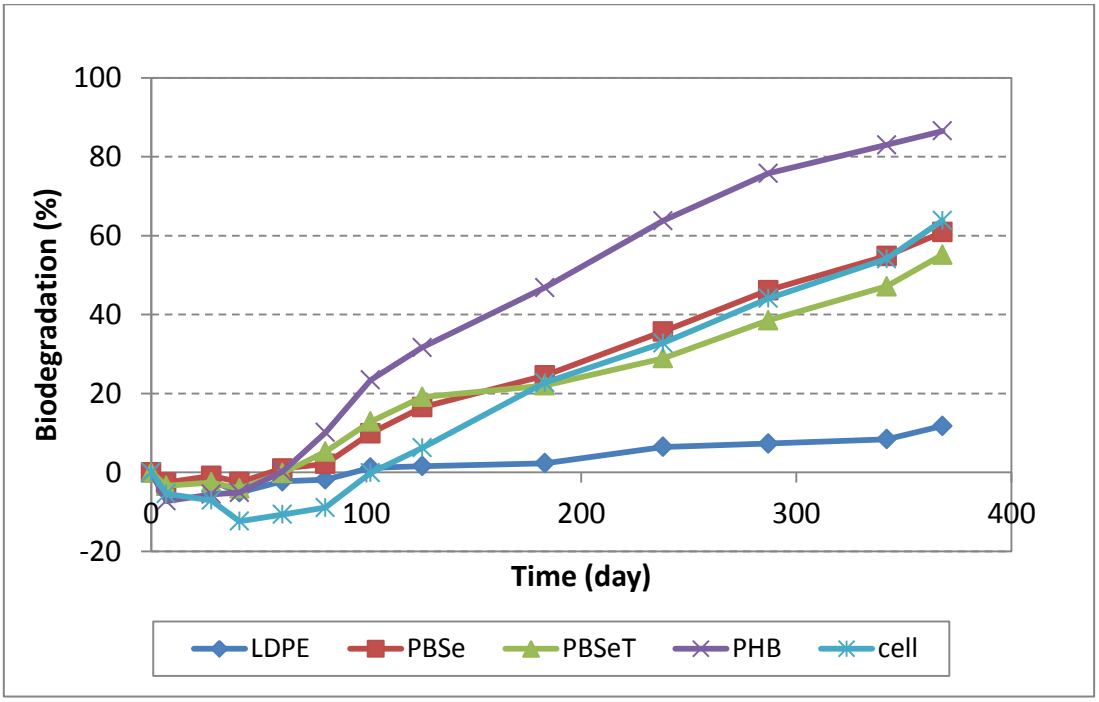


Figure 8. Biodegradation in Eulittoral Environment (Greek Sediment)

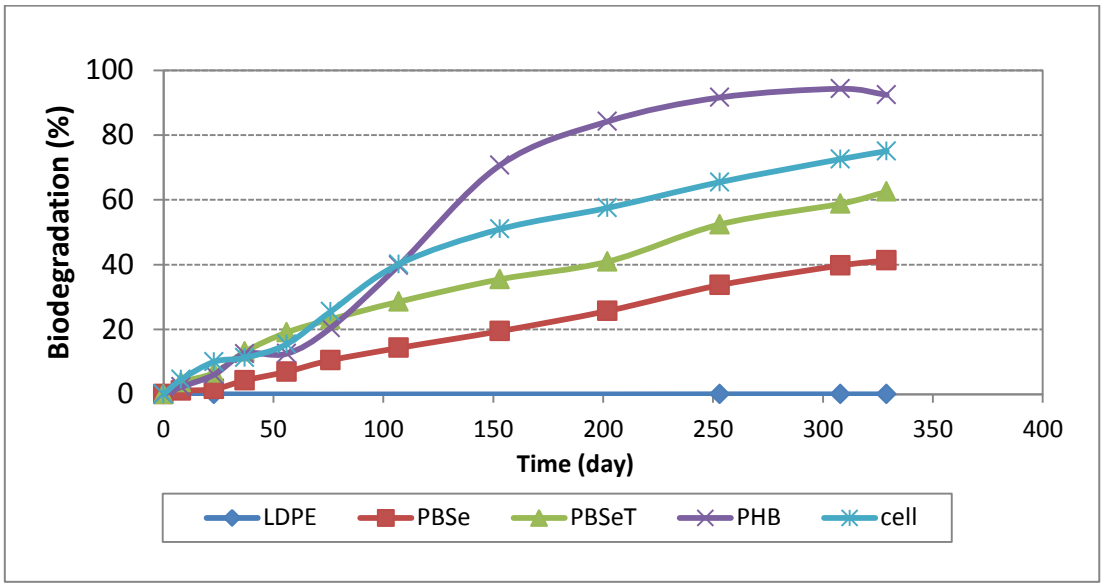


Figure 9. Biodegradation in Eulittoral Environment (Italian Sediment)

Table 8. Biodegradation in Eulittoral environment (Greek Sediment)

Reactor number	Test series	CO_2 cumulative (mg)	Biodegradation (%)	Biodegradation averages
1	Control	176.95	-	-
8	Control	210.03	-	-
10	LDPE	Leakage reactor (defective closure)	-	11.78%
14	LDPE	229.68	11.78	
15	PBSe	335.94	62.08	60.90% (±1.67)
17	PBSe	330.64	59.72	
18	PBSeT	320.74	53.63	55.18% (±2.20)
19	PBSeT	323.93	56.74	
20	PHB	408.06	112.96	86.51%(±37.41)
23	PHB	299.64	60.06	
24	Cellulose paper	337.28	95.56	63.86%(±44.83)
25	Cellulose paper	245.87	32.16	

Reactor 25 showed a very low biodegradation level for cellulose. Normally, the cellulose paper is biodegradable and it is used as positive control in different biodegradation tests. The other replicate (reactor 24) in fact showed a very high biodegradation level. In reactor 24 the cellulose samples disappeared and were invisible in the sediment; in contrast in reactor 25 the samples were visible and recoverable with only little signs of degradation.

Concerning the PHB test material, results highlighted its biodegradability in Eulittoral environment despite very high standard deviation was recorded. Especially, the reactor 20 gives a very high biodegradation (biodegradation > 100%).

Negative control (LDPE test material) shows a low level of biodegradation (11.8%) that is an unexpected results due to this test material is known as totally not biodegradable and the final weight control (retrieved test material) highlighted that no weight loss was happened. These results (PHB > 100% and LDPE > 10%) indicated that an overestimation of CO_2 is present and a consequently it is necessary an improvement of test method.

Table 9. Biodegradation in Eulittoral environment (Italian Sediment)

Reactor number	Test series	CO_2 cumulative (mg)	Biodegradation (%)	Biodegradation averages
1	Control	198.44	-	-
2	Control	275,33	-	-
3	LDPE	242.53	-0.35	0.01%(±0.5)
4	LDPE	244.77	0.36	
5	PBSe	320.32	30.90	41.30%(±14.71)
6	PBSe	368.37	51.70	
7	PBSeT	396.57	60.77	62.55%(±2.52)
8	PBSeT	395.41	64.34	
9	PHB	438.33	112.04*	92.41%(±27.77)
10	PHB	371.01	72.77	
12	Cellulose paper	362.08	75.06	75.06%

*: unrealistic biodegradation value

The results showed in the Table 9 concerning the Italian sediment highlighted the same general behaviour as described before for the Greek sediment. In detail LDPE biodegradation level was closer to zero, PHB and PBSeT showed the same range of biodegradation than in Greek sediment and PBSe obtained a lower biodegradation characterized by a higher standard deviation. In general the biodegradation tendency is confirmed where PHB>PBSe≈PBSeT>LDPE. Also cellulose paper obtained a high degree of biodegradation indicating the validity of the test.

5.1.2 Biodegradation in Benthic zone

The tests were performed according to Annex B and Table 6. The preliminary phase, carried out in order to verify the endogenous respiration in the different reactors and also to oxidize the eventual ready biodegradable organic matter present, was protracted for one week. On the test materials were laid pieces of mosquito net to prevent floating of these. Figure 10, Figure 11, Table 10 and Table 11 summarise the biodegradation trend results.

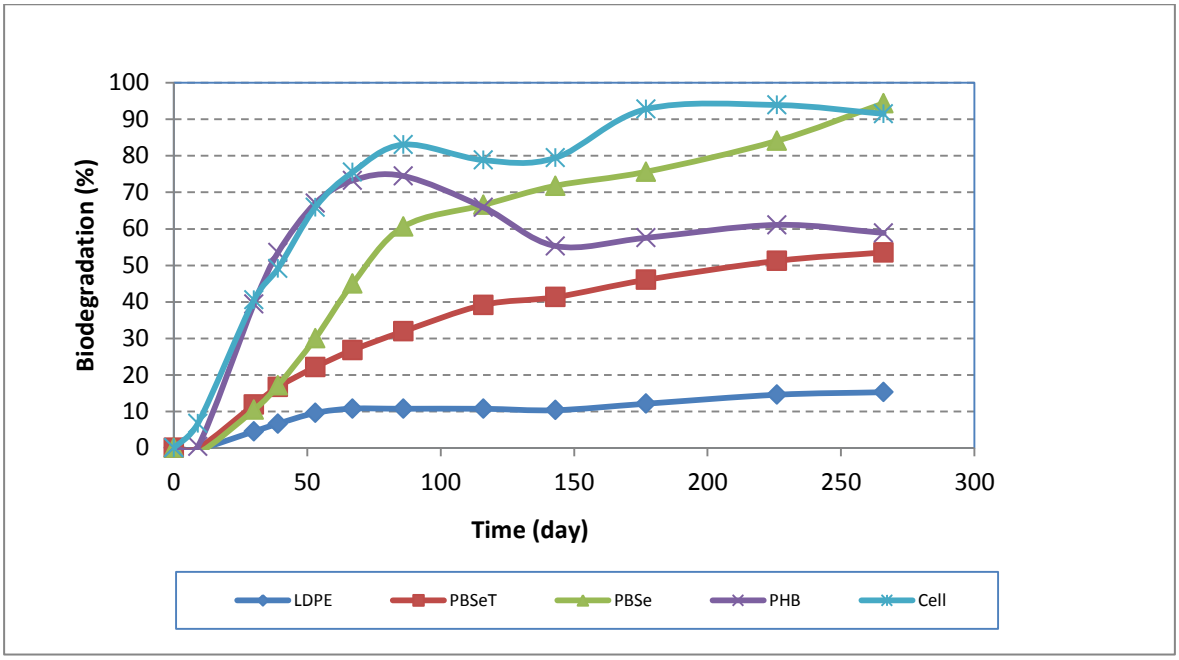


Figure 10. Biodegradation in Benthic environment (Greek inoculum)

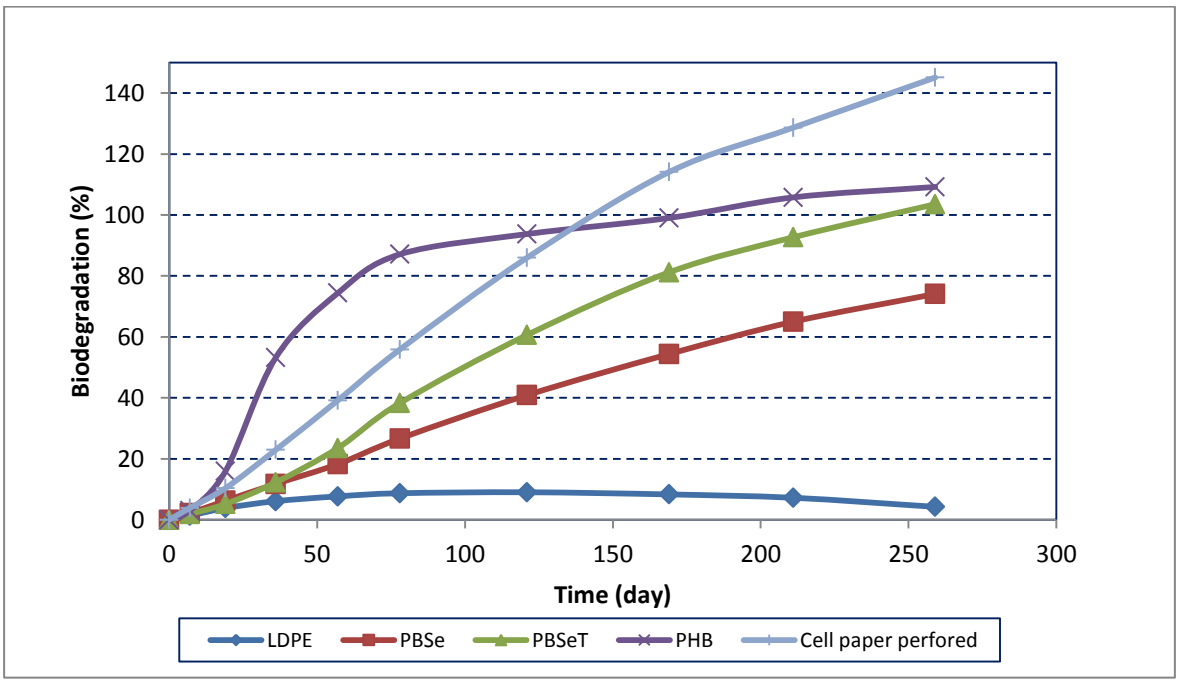


Figure 11. Biodegradation in Benthic environment (Italian inoculum)

Table 10. Biodegradation in Benthic environment (Greek Inoculum)

Reactor number	Test series	CO_2 cumulative (mg)	Biodegradation (%)	Biodegradation Averages
1	Control	142.07	-	-
2	Control	182.05	-	
3	Control	153.41	-	
4	LDPE	169.50	17.50	15.24 (±12.06)
5	LDPE	160.67	2.21	
6	LDPE	174.52	26.02	
7	PBSe	213.20	105.68	94.42 (±12.03)
8	PBSe	207.19	95.82	
9	PBSe	199.57	81.74	
10	PBSeT	175.25	32.67	53.55 (±24.74)
11	PBSeT	197.96	80.88	
12	PBSeT	182.83	47.10	
13	PHB	184.94	62.51	58.89 (±31.39)
14	PHB	194.68	88.30	
15	PHB	169.08	25.85	
16	Cellulose paper	185.25	78.63	91.50 (±60.72)
17	Cellulose paper	211.14	157.63	
18	Cellulose paper	171.40	38.25	
19	Slide Glass	125.02	-	-
20	Slide Glass	155.65	-	

PHB and cellulose paper reached a high level of biodegradation (70%) showing a clear biodegradation phase but with a very large standard deviation. Clear biodegradation was also observed for PBSe. More slowly but constant was the biodegradation of the polymer Polybutylene sebacate-co-butylene terephthalate (PBSeT). The negative reference material LDPE showed a biodegradation of 10%, which is difficult to explain. One hypothesis about this strange behaviour, was the limitation of the oxygen exchange with the establishment of anaerobic conditions, with CH_4 and CO_2 production, from anaerobic degradation of the organic matter present in the sediment. Probably this over production of CO_2 was measured. By using glass slides we tried to maximize this effect; the slides are more heavy than the plastic film, resulting in a good contact with the sediment and naturally the glass was not degradable. In Table 10 the CO_2 production of the different replicates, the biodegradation levels and the standard deviations are reported. The CO_2 production (average) in the reactors with the slides was 151 mg (±23.6) while in control reactors (only sediment) was 159 (±20.6). This results seem showed as the over production of CO_2 observed in reactors with LDPE and also in one reactor with cellulose is not linked to a limitation of oxygen exchanged and consequently the establishment of anaerobic condition

but probably to the high amount of organic matter present in the sediment (about 4% of volatile solids) and the insufficient stabilization of this during the preliminary phase.

Table 11. Biodegradation in Benthic Environment (Italian inoculum)

Reactor number	Test series	CO_2 cumulative (mg)	Biodegradation (%)	Biodegradation Averages
1	Control	74.91	-	-
2	Control	74.66	-	
3	Control	77.55	-	
4	LDPE	81.98	9.82	4.26 (±6.29)
5	LDPE	74.06	-2.56	
6	LDPE	79.24	5.52	
7	PBSeT	120.30	86.60	103.52 (±23.13)
8	PBSeT	119.29	94.09	
9	PBSeT	141.93	129.88	
10	PBSe	114.58	78.89	74.08 (±5.47)
11	PBSe	107.87	68.13	
12	PBSe	113.13	75.23	
13	PHB	114.68	111.81	109.19 (±19.95)
14	PHB	123.83	127.71	
15	PHB	108.38	88.07	
16	Cellulose paper	143.99	160.51	145.09 (±15.43)
17	Cellulose paper	130.92	129.64	
18	Cellulose paper	131.06	145.13	
19	LDPE perforated	96.99	33.17	47.62 (±14.98)
20	LDPE perforated	104.29	46.62	
21	LDPE perforated	118.60	63.08	

Once again a very large standard deviation were observed. An over production of CO_2 was measured in the reactors with cellulose. In this experiment in order to improve the oxygen exchange, three reactors with perforated LDPE were prepared. The LDPE film was perforated manually using a circular die with a diameter of 4 mm. Surprisingly a very high over production of CO_2 was measured and the LDPE samples were recovered intact at the end of the test.

5.1.3 Biodegradation in Pelagic zone

Before the start-up nutrients KH_2PO_4 (0.1 g/L) and NH_4Cl (0.05 g/L) were added to the seawater after discussion with other project partners. They serve as nutrients for the micro-organisms, which stand in for the biodegradation of the specimens. In the pelagic test any preliminary phase was carried out.

Novamont prepared three different tests:

- Biodegradation in Greek seawater with determination of CO_2 evolved. Two replicates per test material, two blank controls. Test temperature: 25°C (±2).

- Biodegradation in Greek seawater with determination of O_2 consumed. Three replicates per test material, three blank controls. Test temperature: 28°C (±2).

- Biodegradation in Italian seawater with determination of CO_2 evolved. Three replicates per test material, three blank controls. Test temperature: 28°C (±2).

The tests were performed according to ASTM D6691 and Table 6. The test items were milled to powders using liquid nitrogen. A plastic container with 3 ml KOH 0,5 M was added in the test with determination of CO_2, while the Oxytop® system was used for the determination of biodegradation measuring the oxygen consumption and trapping the CO_2 produced with a KOH solution (in this specific case 1 N) that it was possible to titrate during the test in order to have a double check of the biodegradation reactions: oxygen consumption and CO_2 development. The system based on determination of CO_2 had very bad results, it was assumed that the reason is due to the equipment used. The seawater was in agitation during the test, and the plastic container with the KOH was suspended in the reactor. Probably a small splash of seawater has entered in the container and has polluted/diluted the KOH solution. To improve the system it is necessary to protect the KOH container as happens in Oxytop system, or to use a different approach with a dynamic system where a decarbonated air flow passes through the reactor and the CO_2 evolved is caught in a trap with barium hydroxide as described in ISO 14852. On the contrary, good results were obtained using the Oxytop system, both the measures of oxygen consumption that the CO_2 evaluated. Figure 12 and Figure 13 show the biodegradation curves, and Table 12 shows the results.

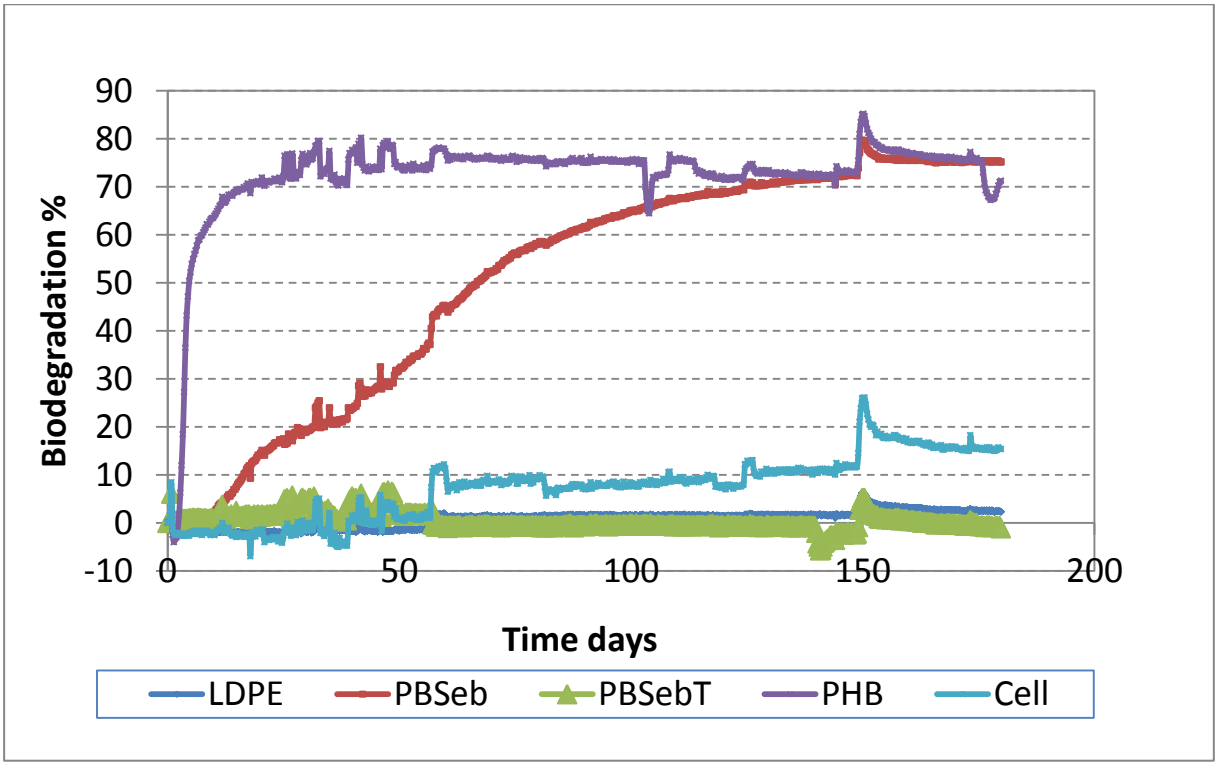


Figure 12. Biodegradation of test materials in Pelagic zone simulation. Greek inoculum – Oxytop System

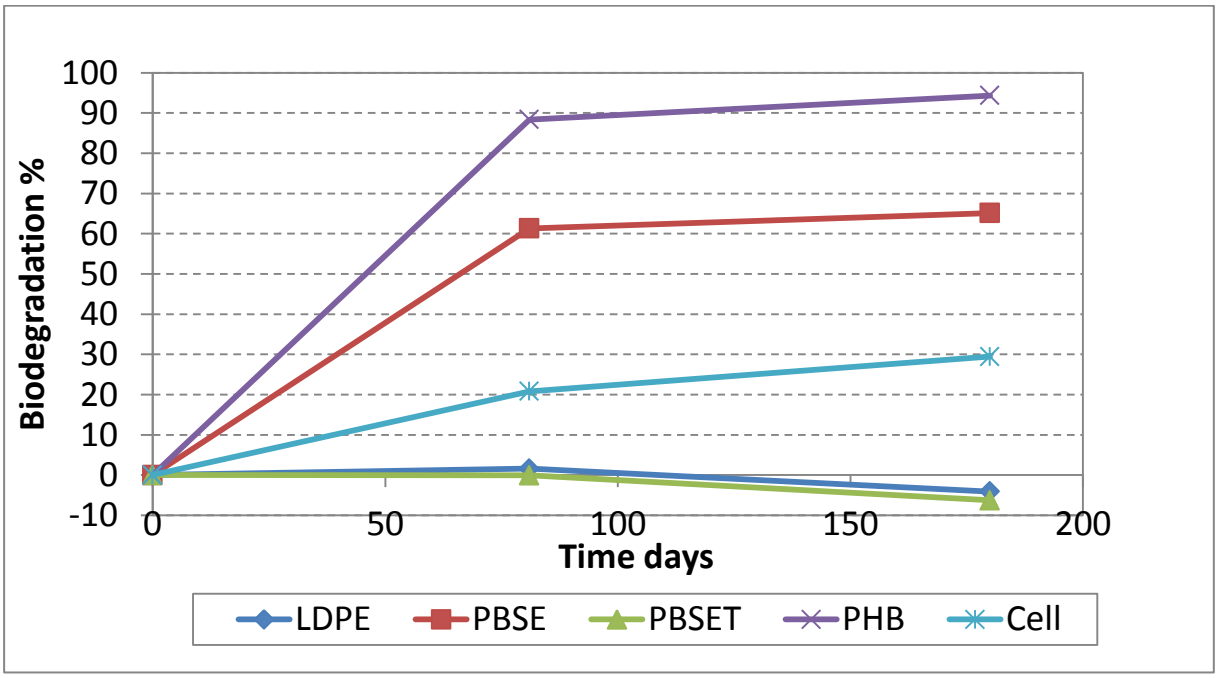


Figure 13. Biodegradation of test materials in Pelagic zone simulation. Greek inoculum - Determination of CO_2 during the Oxytop test

Table 12. Final results biodegradation in seawater from Greece

Test material	Biodegradation (%)	Biodegradation Average (%)	Biodegradation average based on CO_2 evolution (%)
LDPE	0.88	2.29 (±1.25)	-4.07
LDPE	2.69		
LDPE	3.28		
PBSe	63.63	75.22 (±10.43)	65.15
PBSe	83.86		
PBSe	78.16		
PBSeT	-1.54	-1.16 (±4.40)	-6.26
PBSeT	-5.35		
PBSeT	3.41		
PHB	87.22	71.23 (±13.85)	94.35
PHB	63.19		
PHB	63.29		
Cellulose Paper	16.31	15.41 (±0.89)	29.44
Cellulose Paper	14.53		
Cellulose Paper	15.41		

No biodegradation was observed in PBSeT and LDPE, high biodegradation level was reached by PHB and PBSe. The cellulose in all reactors showed only a partial biodegradation, which is an unexpected result because the cellulose is normally used as positive control in biodegradation tests and also in ASTM D6691. On the contrary the biodegradation of PHB, that we can consider another positive control, was high as expected. The recommendation for further experiments is to include the cellulose in order to confirm or not this result. Laboratory: OWS

5.2 Laboratory: OWS

5.2.1 Biodegradation in Eulittoral zone

A sandy sediment has been withdrawn from the eulittoral zone of the shoreline in Greece and Italy by respectively the Agricultural University of Athens (AUA) and Hydra Institute for Marine Science, where the sediment is submerged by sea water at times other than low tide. Figure 14 gives an image of the received sediment of Greece, while Figure 15 shows the sediment of Italy.

Figure 14. Visual presentation of the filtration procedure (Greece)

Figure 15. Visual presentation of the filtration procedure (Italy)

For the preliminary phase 400 g of the sandy sediment has been weighted and placed on the bottom of a reactor in the form of a homogenous layer. Then, a container with KOH (the CO_2 trapping solution) was added to each reactor. Every reactor also contained a beaker distilled water. The function of the beaker water was to prevent dehydration of the sediments. Subsequently, these reactors have been placed in a chamber kept at a temperature of 28°C. In order to verify the similarity of endogenous respiration in the different reactors, the CO_2 was regularly monitored by means titrating the KOH solution with HCl. Another importance of this preliminary phase is oxidation of excess organic matter, ensuring to start the test with a lower endogenous respiration. This phase is protracted for a week. For the sediment of Greece, a titration was performed four days after start-up. By performing a Q-test (99% confidential interval) on the CO_2 levels of the reactors, it was possible to conclude that these reactors had enough similarity to start the test. For the sediment of Italy a preliminary phase of six days was performed. Judging from the first titration, all reactors had enough similarity to start the test. In total, 18 reactors (each with a 4 L volume) were prepared. Three reactors for each specimen (4 specimens), 3 control reactors and 3 technical control reactors. After the preliminary phase, 100 g of the sediment was removed (see Figure 17) from the top layer in the bottom of the reactor. The surface of the residual sediment has been made smooth with a spatula and the specimens were placed on top of the residual sediment, which can be seen in Figure 16 (not applied for the blank reactors). Consequently, the withdrawn sediment was replaced back in the reactors to form a homogenous layer that covers the specimens. These are in the form of a film or plate, cut in squares with a length of approximately 4-5 cm and a mass of 100 mg. Samples LDPE, PBSe and PBSeT were cut into 4 cm × 4 cm pieces. Two 4 cm × 4 cm pieces of these materials correspond to approximately 100 mg. The test material PHB was cut into smaller pieces for its high thickness.

Figure 16. Visual presentation of 100 g removed sediment (Greece)

Figure 17. Visual presentation of the specimen (Polyhydroxy alcanoate) on the residual sediment (Greece)

The period of time between the carbon dioxide analysis by means of titrations were variable. Titrations were performed with the recommended frequency of every 3 to 4 days the first 2 weeks and once every 2 weeks thereafter, for the test with the sediment of Greece. Because of the low biodegradability of the test items and low background activity of the sediment, titrations were performed once every 2 weeks for the test with the sediment of Italy (even at start). When the KOH solutions were removed, the moisture loss from the sediment has been monitored by means of measuring the reactors' weights. When moisture loss was too abundant, distilled water was added to the sediment until original weight of the reactor was achieved. Before placing a new KOH container, the reactors remained open for about 15 minutes allowing the air in the reactors to refresh. At the start of the test with the sediment of Greece, a solution of 30 mL KOH (1 N) was chosen, which was titrated with 1 N HCl. Due to the inaccurate results, after 2 titrations a solution of 10 mL KOH (0.5 N) was used (which was titrated with 0.1 N HCl). Because the test with the sediment of Italy was started at a later date, solutions of 10 mL KOH (0.5 N) and 0.1 N HCl were used from the start of the test. Figure 18 shows the evolution of the biodegradation of the different samples in sandy marine sediment from Greece. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation was observed for PHB copolymer, followed by PBSe and PBSeT. Negligible biodegradation was observed for LDPE. At the end of the test (= after 425 days), PHB copolymer has reached a biodegradation percentage of 61.5% ± 10.9%. The biodegradation percentages of the other samples were 5.4% ± 5.2% for LDPE, 23.0% ± 12.3% for PBSe and 14.2% ± 8.5% for PBSeT. The evolution of the net cumulative CO_2 production in the blank reactors have reached a net cumulative CO_2 production of 125 mg ± 10 mg. The net cumulative CO_2 production of the samples were 141 mg ± 15 mg for LDPE, 235 mg ± 19 mg for PBH copolymer, 178 mg ± 28 mg for PBSe and 162 mg ± 24 mg for PBSeT.

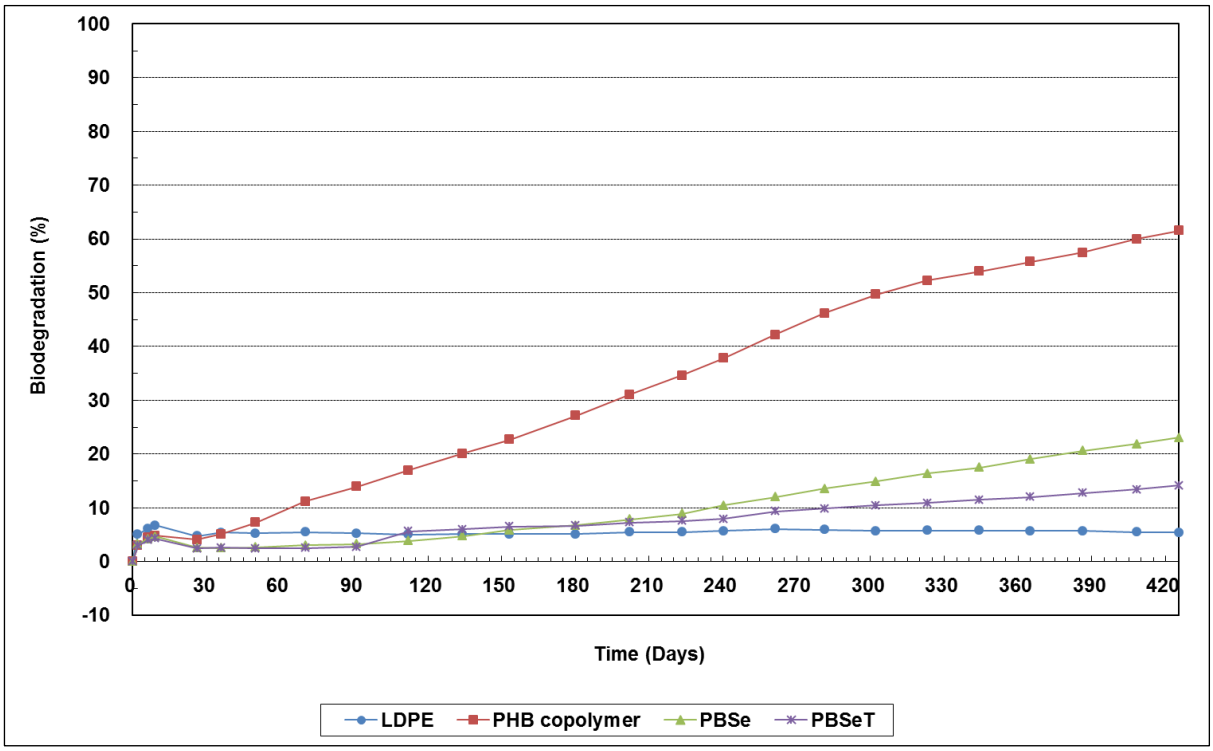


Figure 18. Evolution of biodegradation in sandy marine sediment (Greece)

The evolution of the net cumulative CO_2 production of the individual replicates is given in Figure 19 up to Figure 23. At the end of the test (= after 425 days), the blank reactors have reached a net cumulative CO_2 production of 125 mg ± 10 mg. The net cumulative CO_2 production of the samples were 141 mg ± 15 mg for LDPE, 235 mg ± 19 mg for PBH copolymer, 178 mg ± 28 mg for PBSe and 162 mg ± 24 mg for PBSeT. The graph of PBSeT reveals that one titration was most probably incorrect. In case this titration would be neglected a biodegradation percentage of 11.3% ± 4.4% would be obtained after 425 days for PBSeT instead of 14.2% ± 8.5%.

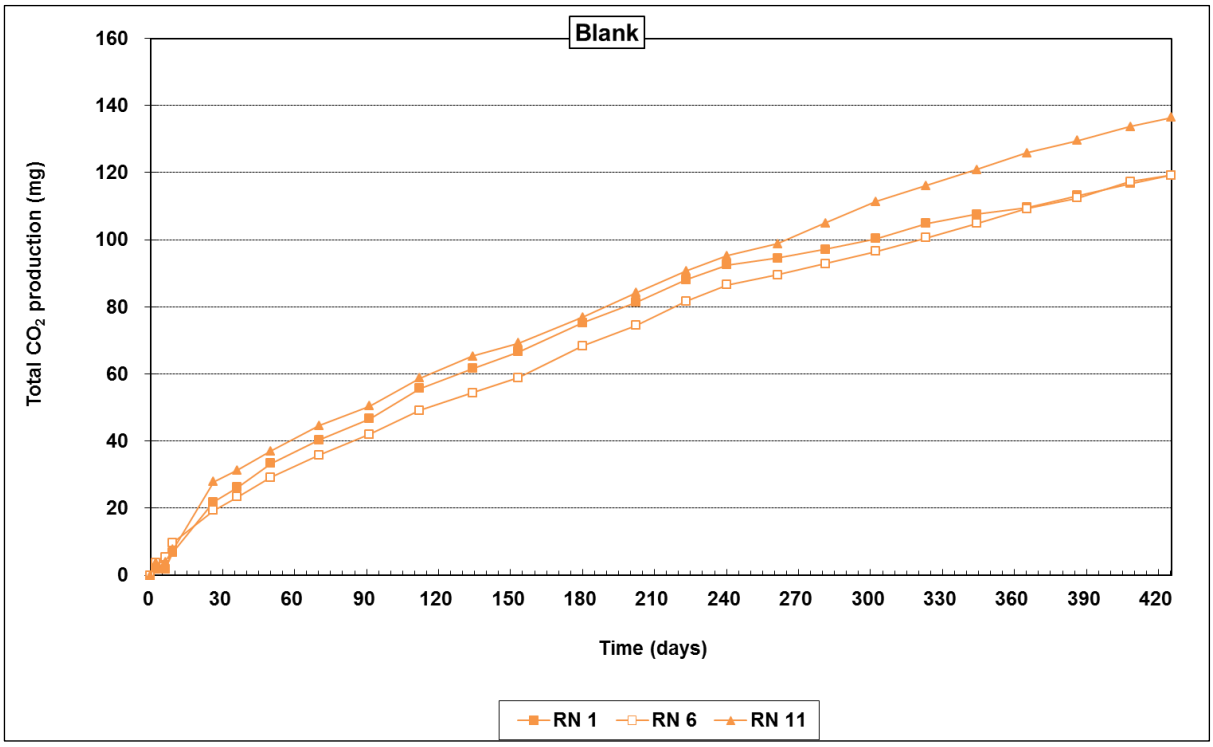


Figure 19. Total CO_2 production in blank reactors in sandy marine sediment (Greece)

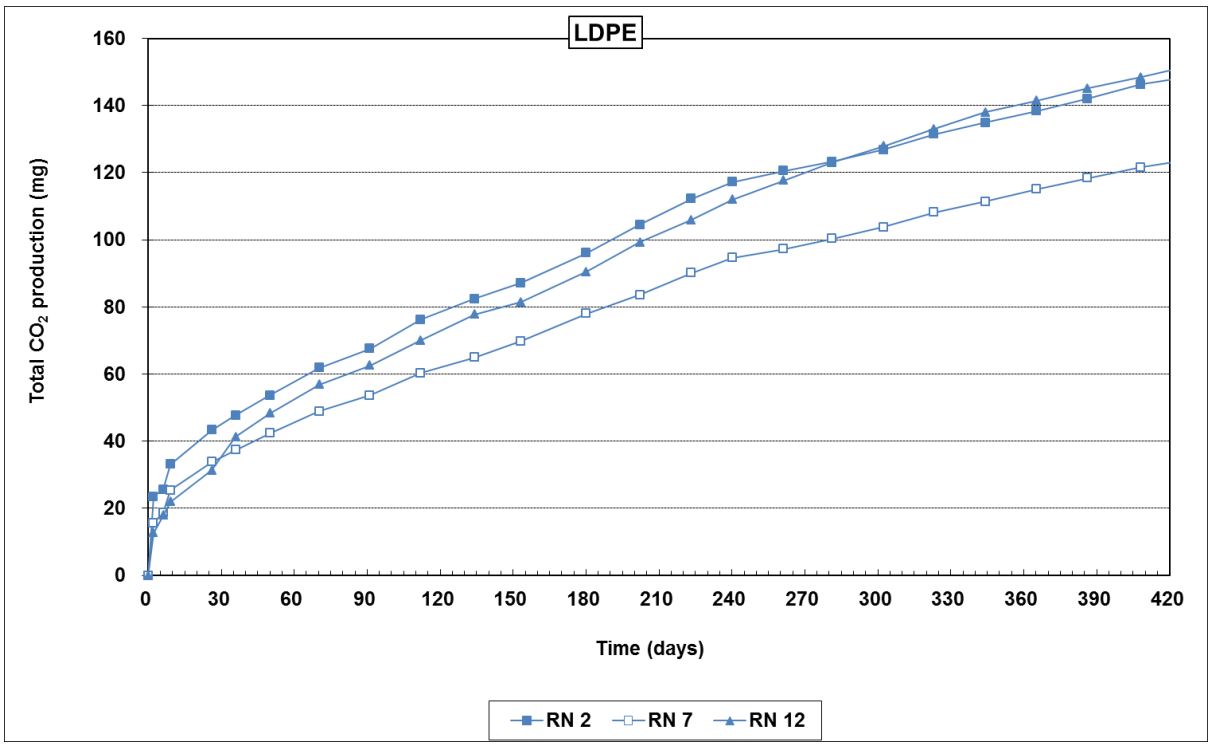


Figure 20. Total CO_2 production in LDPE reactors in sandy marine sediment (Greece)

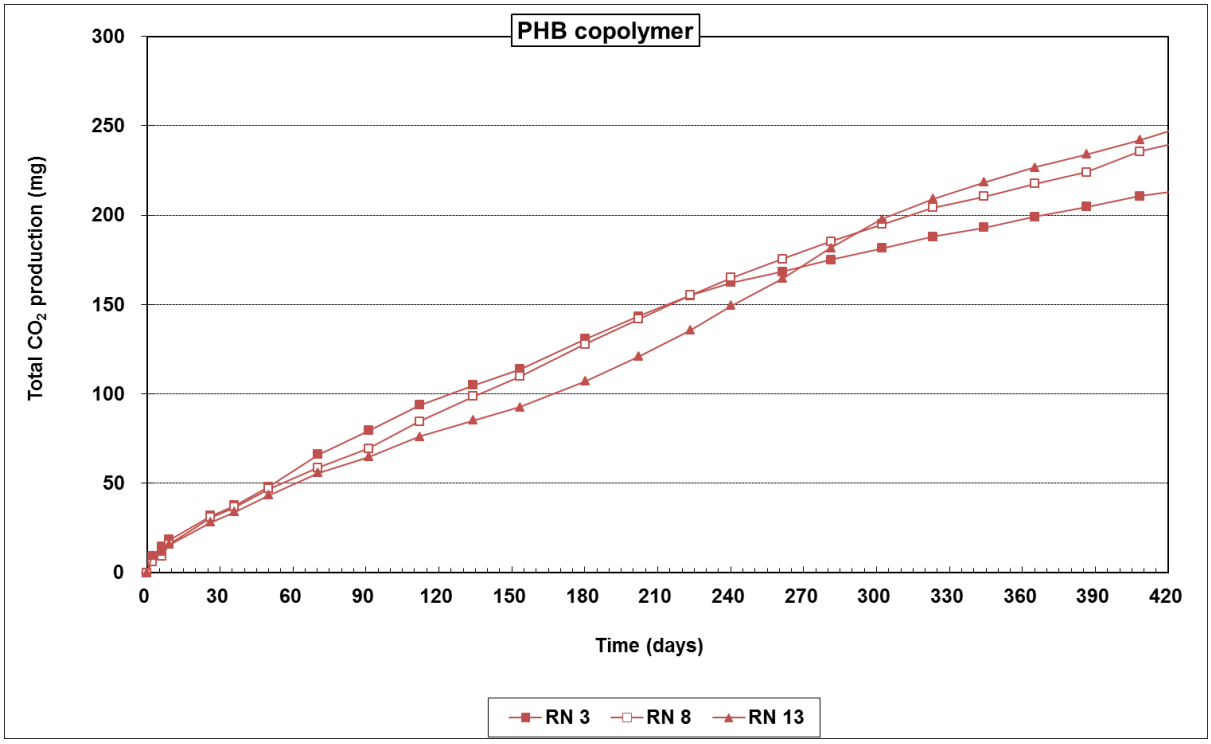


Figure 21. Total CO_2 production in PHB reactors in sandy marine sediment (Greece)

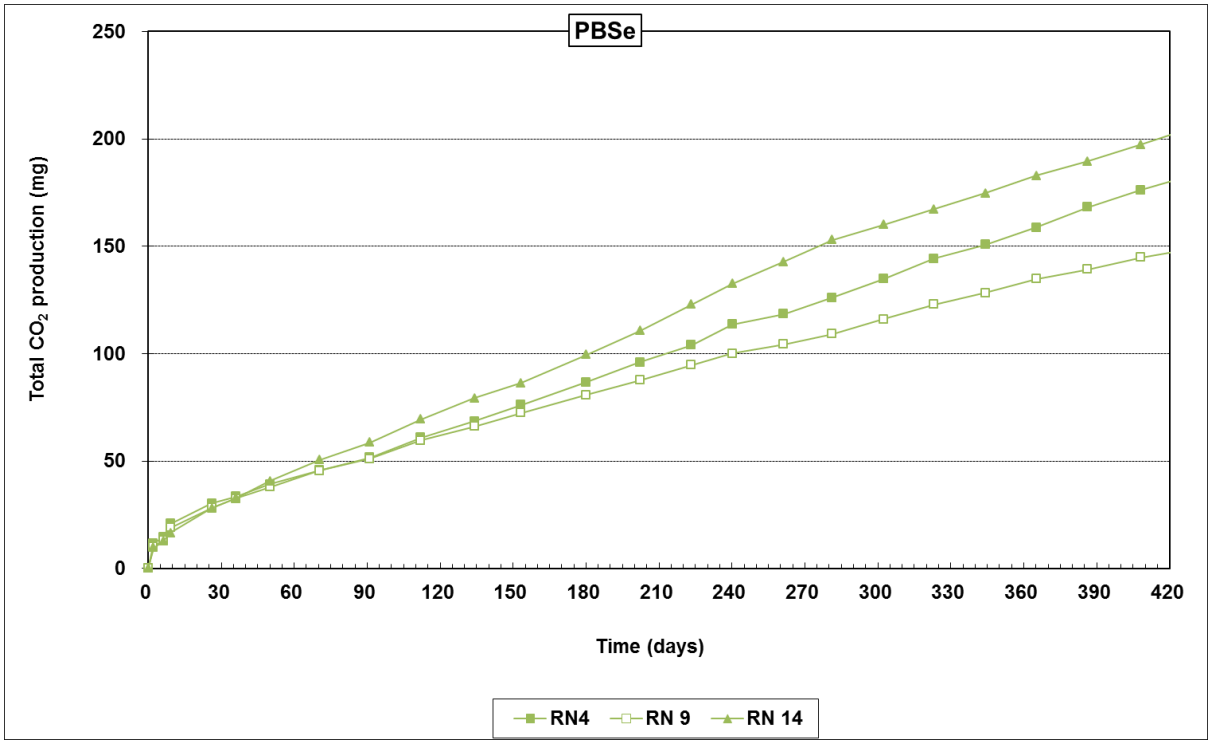


Figure 22. Total CO_2 production in PBSe reactors in sandy marine sediment (Greece)

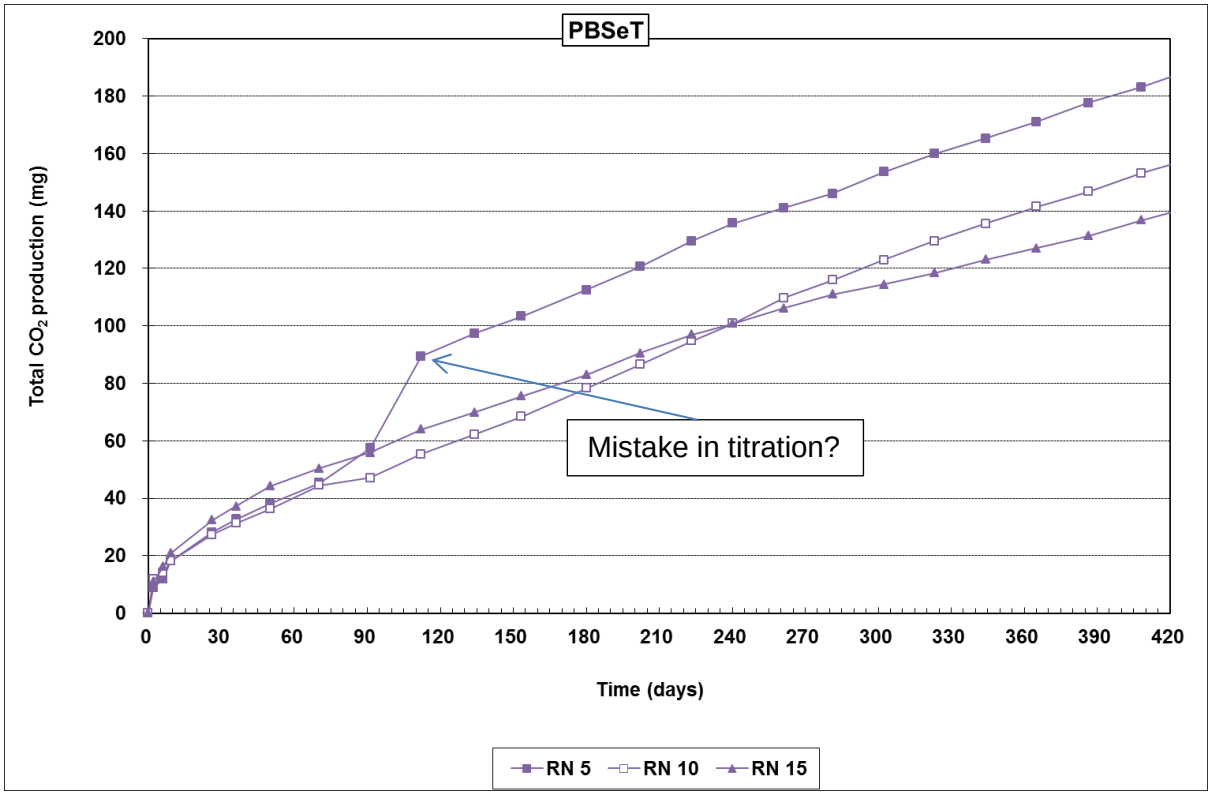
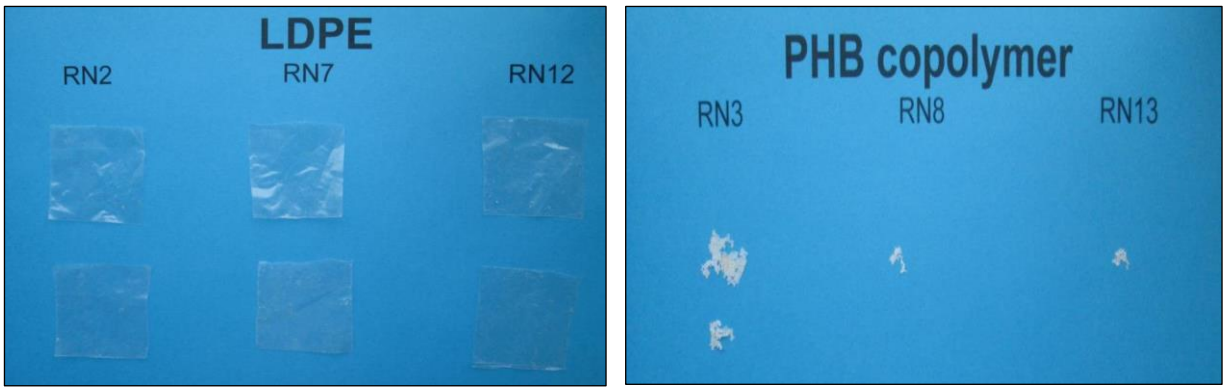


Figure 23. Total CO_2 production in PBSeT reactors in sandy marine sediment (Greece)

At the end of the test (= after 425 days) the remaining test material was manually retrieved, dried and weighted. An overview of the disintegration percentages is given in Table 13, in Figure 24 and Figure 25. The disintegration follows the same pattern as the biodegradation (PHB copolymer >> PBSe > PBSeT > LDPE).



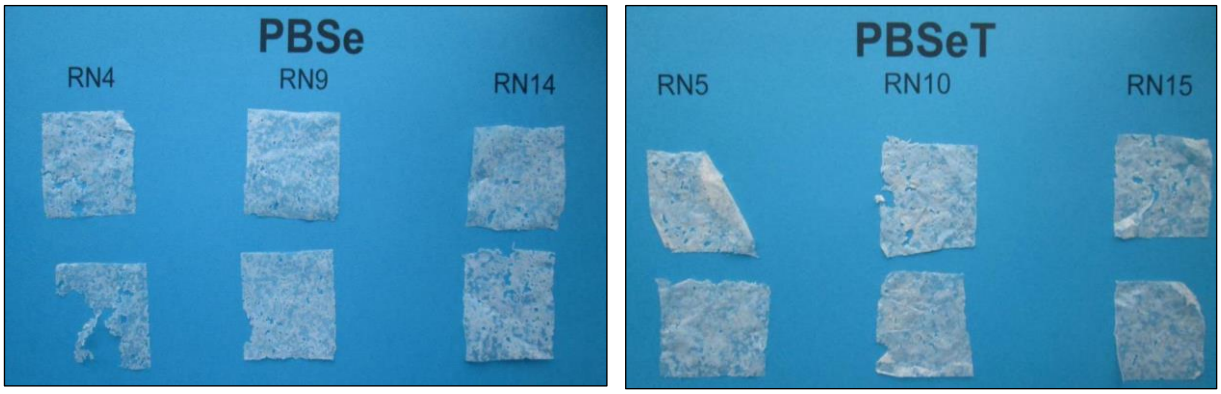


Figure 24. Overview of retrieved pieces of LDPE, PHB, PBSe and PBSeT

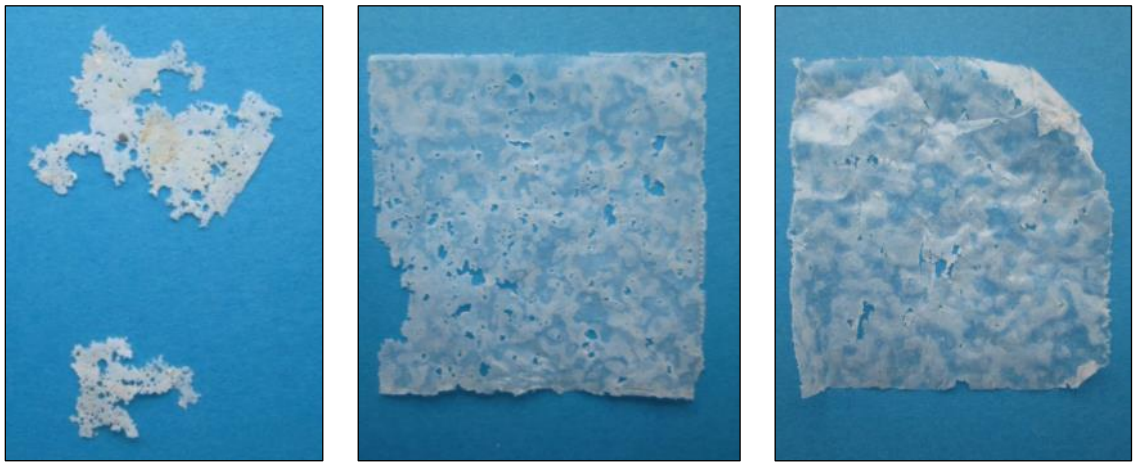


Figure 25. Detailed pictures of PHB copolymer (left), PBSe (middle) and PBSeT (right)

Table 13. Description and disintegration percentage of retrieved test materials after 425 days

Test item	Description	Disintegration	Biodegradation
LDPE	Intact	-3% ± 4%	5.4% ± 5.2%
PHB	Only small pieces were retrieved Strongly fragmented	95% ± 6%	61.5% ± 10.9%
PBSe	A lot of small holes were present in the 4 cm × 4 cm pieces	23% ± 9%	23.0% ± 12.3%
PBSeT	Small holes were present in the 4 cm × 4 cm pieces	17% ± 4%	14.2% ± 8.5%

Figure 26 shows the evolution of the biodegradation of the different samples in sandy marine sediment from Italy. It was possible that the biodegradation rate was low for PBSe and PBSeT due to deficiency of nutrients. After 323 days of incubation, it was decided to add 0.1 mg N/mg C of test item. No significant difference in biodegradation rate was observed due to the addition of nutrients. The difference in biodegradation pattern between the samples is rather logical. The fastest biodegradation was observed for PHB copolymer, followed by PBSe and PBSeT, which were characterized by a similar biodegradation (in contrast to the sediment from Greece in which a higher biodegradation was reached for PBSe when compared to PBSeT). Negligible biodegradation was observed for LDPE. At the end of the test (= after 393 days), PHB copolymer has reached a biodegradation percentage of 96.2% ± 3.1%. The biodegradation percentages of the other samples were 3.5% ± 4.1% for LDPE, 26.6% ± 3.2% for PBSe and 27.0% ± 7.2% for PBSeT.

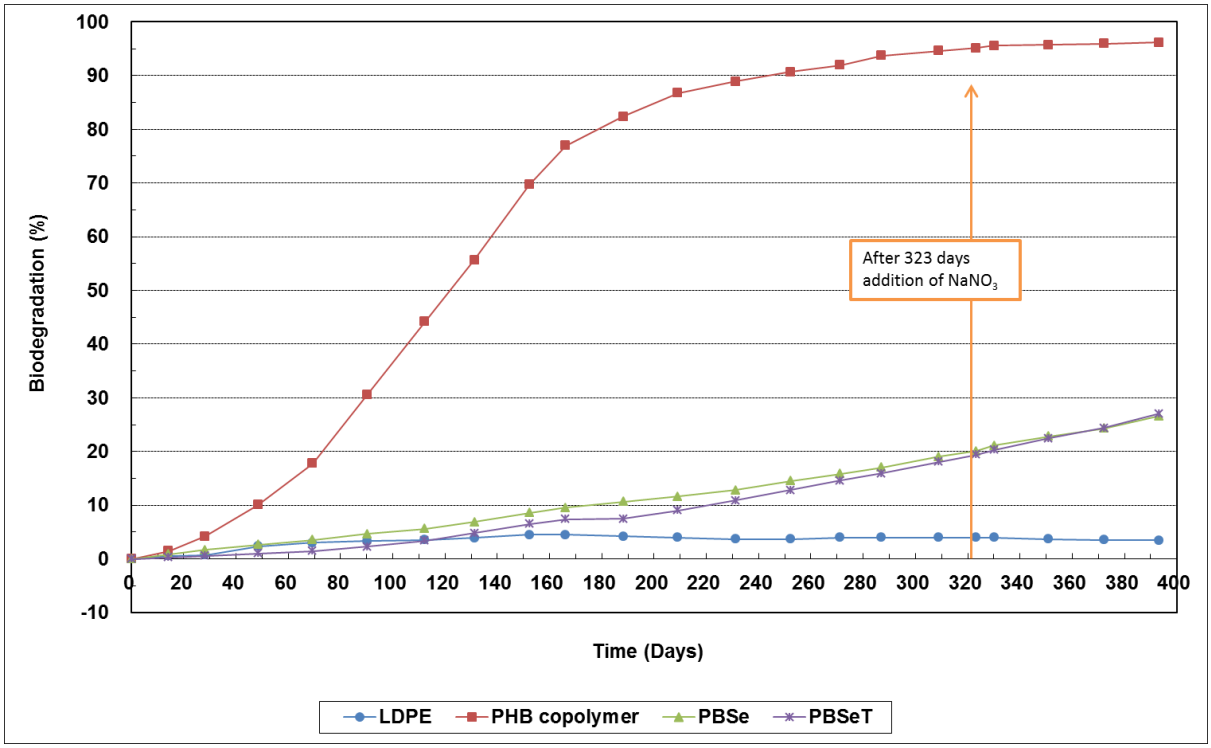


Figure 26. Evolution of biodegradation in sandy marine sediment (Italy)

The net cumulative CO_2 production in the blank reactors was 128 mg ± 6 mg. The net cumulative CO_2 production of the samples was 138 mg ± 12 mg for LDPE, 286 mg ± 4 mg for PBH copolymer, 187 mg ± 7 mg for PBSe and 193 mg ± 16 mg for PBSeT. The variability in the total CO_2 production between the three replicates is lower in the sandy sediment from Italy when compared to the sandy sediment from Greece. The sand from Italy was characterized by a fine structure, while more coarse particles were present in the sand of Greece. This might be the reason for the difference in variability. At the end of the test (= after 425 days) the remaining test material was manually retrieved, dried and weighted. An overview of the disintegration and also the biodegradation percentages is given in Table 14.

The disintegration follows the same pattern as the biodegradation (PHB >> PBSe > PBSeT > LDPE).The evolution of the total CO_2 production of the individual replicates is given up Figure 27 to Figure 31. At the end of the test (= after 393 days), the blank reactors have reached a net cumulative CO_2 production of 128 mg ± 6 mg. The net cumulative CO_2 production of the samples were 138 mg ± 12 mg for LDPE, 286 mg ± 4 mg for PHB copolymer, 187 mg ± 7 mg for PBSe and 193 mg ± 16 mg for PBSeT. The variability in the total CO_2 production between the three replicates is lower in the sandy sediment from Italy when compared to the sandy sediment from Greece. The sand from Italy was characterized by a fine structure, while more coarse particles were present in the sand of Greece. This might be the reason for the difference in variability.

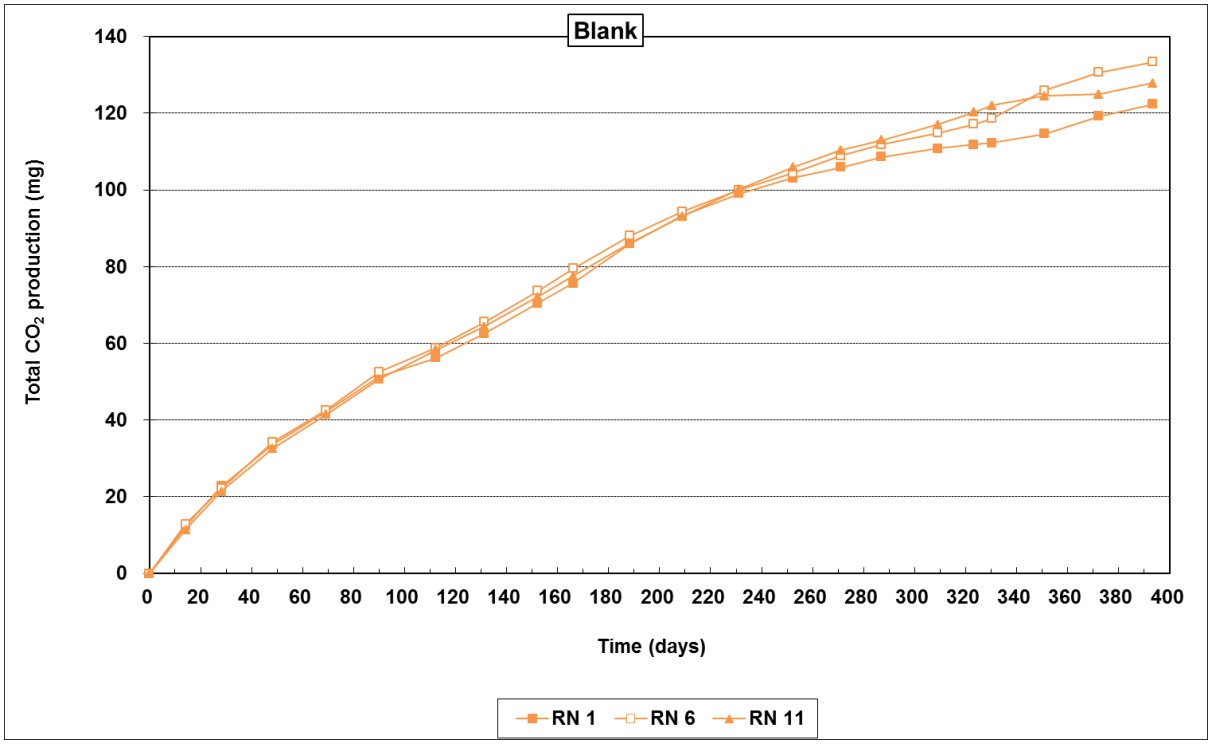


Figure 27. Total CO_2 production in blank reactors in sandy marine sediment (Italy)

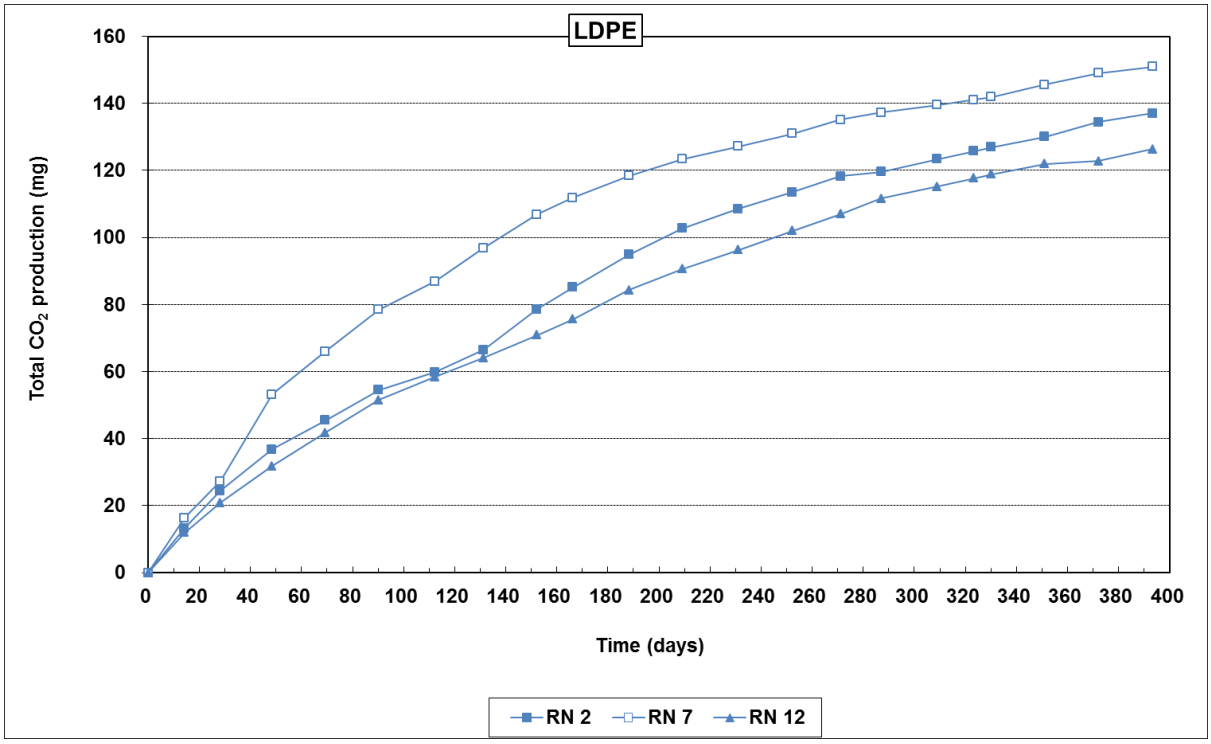


Figure 28. Total CO_2 production in LDPE reactors in sandy marine sediment (Italy)

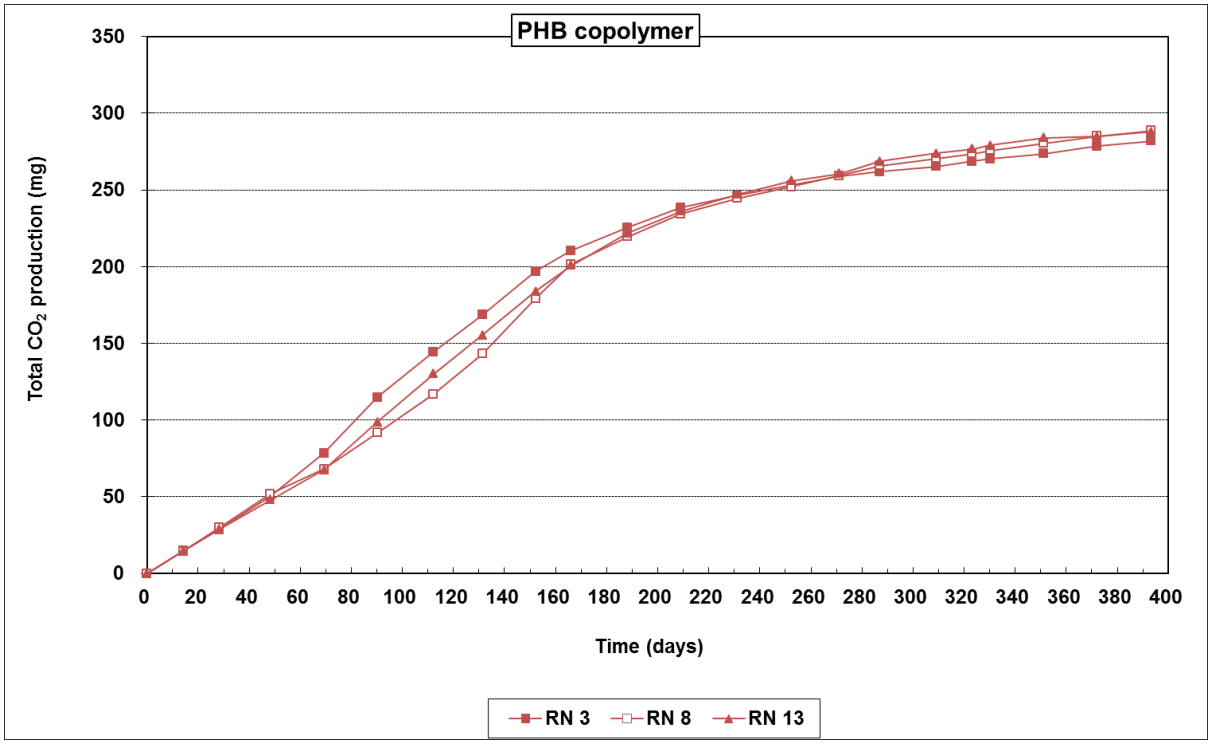


Figure 29. Total CO_2 production in PHB reactors in sandy marine sediment (Italy)

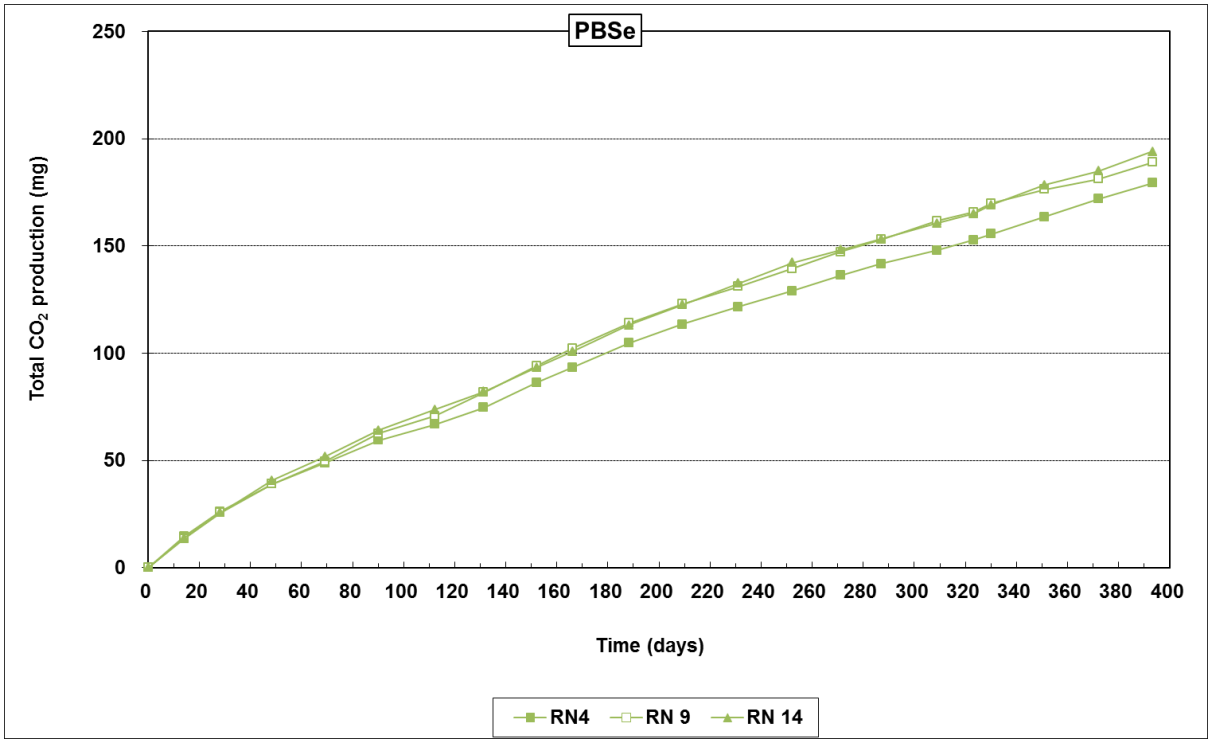


Figure 30. Total CO_2 production in PBSe reactors in sandy marine sediment (Italy)

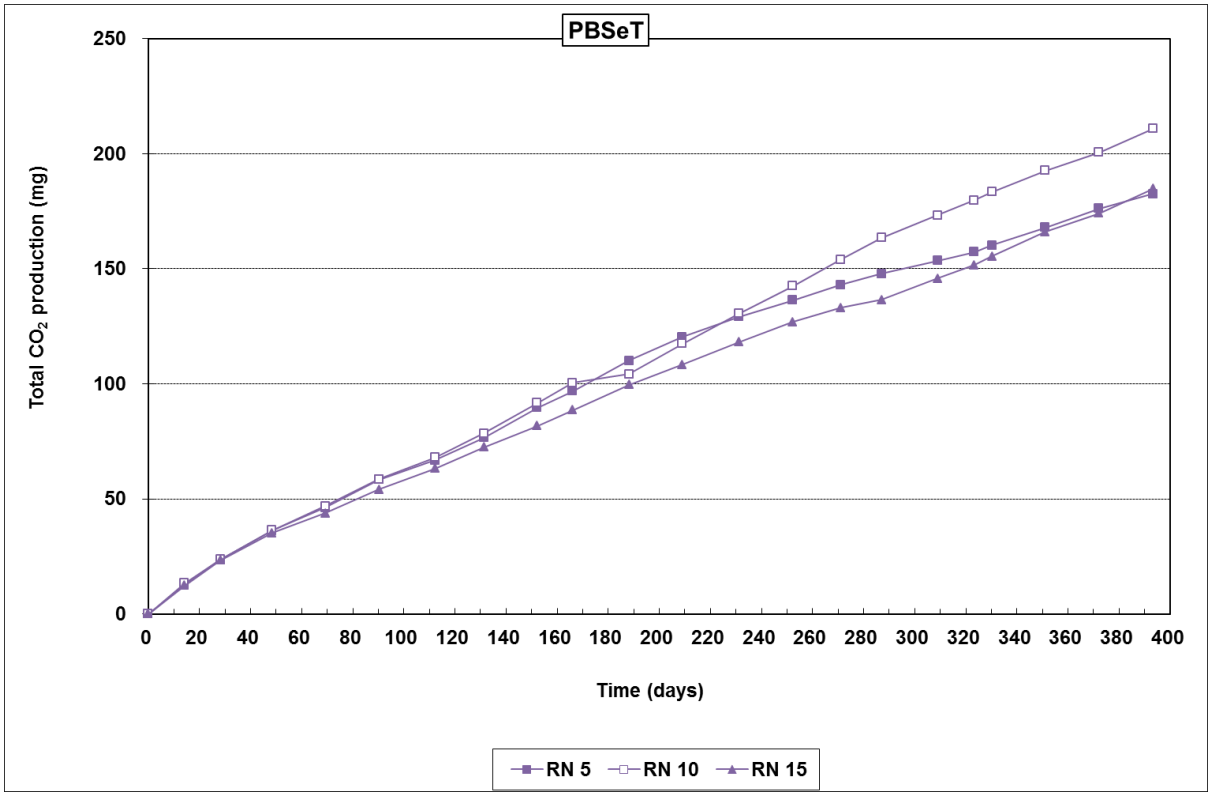


Figure 31. Total CO_2 production in PBSeT reactors in sandy marine sediment (Italy)

At the end of the test (= after 393 days) the remaining test material was manually retrieved, dried and weighted. An overview of the weights is given in Table 13. The retrieved pieces are shown in Figure 32.

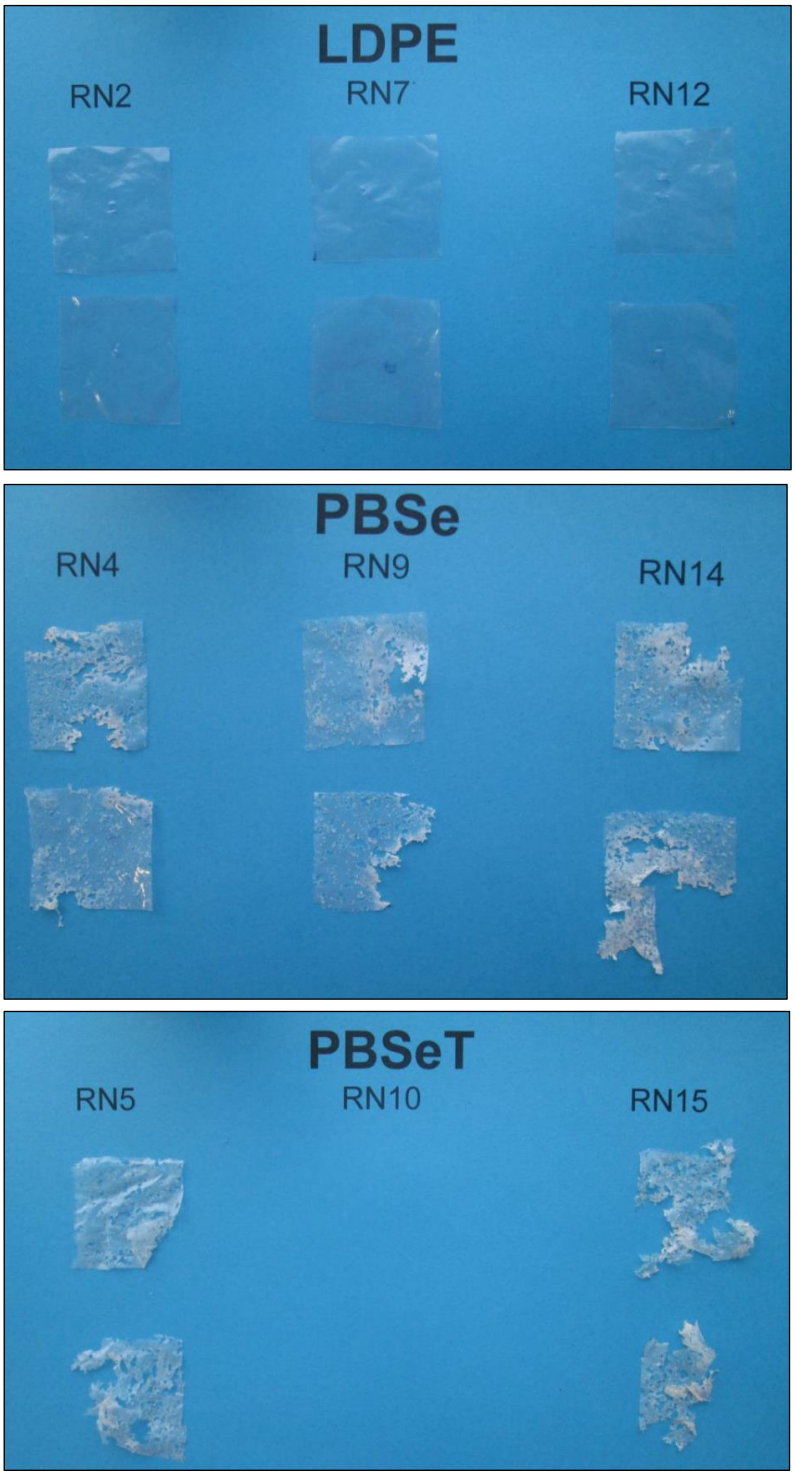


Figure 32. Overview of retrieved pieces of LDPE, PBSe and PBSeT

Table 14. Description and disintegration percentage of retrieved test materials after 393 days

Test item	Description	Disintegration	Biodegradation
LDPE	Intact	-3% ± 4%	3.5% ± 4.1%
PHB	Only small pieces were retrieved Strongly fragmented	95% ± 6%	96.2% ± 3.1%
PBSe	A lot of small holes were present in the 4 cm × 4 cm pieces	23% ± 9%	26.6% ± 3.2%
PBSeT	Small holes were present in the 4 cm × 4 cm pieces	17% ± 4%	27.0% ± 7.2%

The comparison of the results in Figure 33. LDPE, PBSe and PBSeT had a similar biodegradation percentage in both sediments. PHB copolymer has reached 90% biodegradation in the sediment from Italy, but in the sediment from Greece only a biodegradation percentage of 60% was obtained.

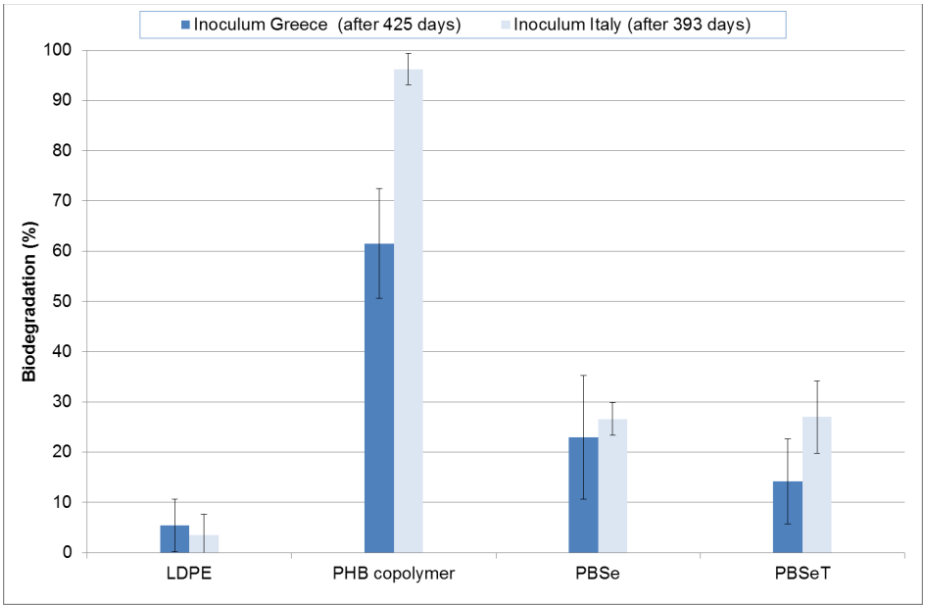


Figure 33. Final biodegradation level comparison between the Greek and Italian inoculums

Suggestion 1: In the current test set-up approximately 100 g of sandy sediment needs to be removed from the reactor. Subsequently sample needs to be added and then the 100 g of sandy sediment again needs to be placed on the samples. Perhaps it would be better if the samples are closer to the surface of the sediment (for example: remove only 25 g sandy

sediment or just cover the sample with a thin layer sand). In this way the environment would be characterized by a higher oxygen content.
Suggestion 2: Increase the amount of sample. A sediment test could be compared with a biodegradation test in soil (ISO 17556). A maximum amount of 5 g is allowed per 400 g soil.

5.2.2 Biodegradation in Benthic zone

Seawater and a sandy sediment have been sampled at a great distance of the shoreline in Greece and in Italy, respectively by the Agricultural University of Athens and by Hydra Institute for Marine Science. The sediment and seawater were transported to Belgium. At arrival the seawater and sediment were stored at approximately 4°C. Obvious plant material, sea shell, pieces of driftwood, or other large pieces of material were removed from the sandy sediment. Before the start set-up, the seawater and the sediment were separated by filtering it through a funnel using a fine grid. For the preliminary phase, 30 g sediment and 70 mL seawater have been measured and put in the test flasks (see Table 6). An intermediary phase needed to be established between the sediment and the seawater. An homogenous interphase was obtained at the bottom of a reactor between the sediment and the seawater. Subsequently, 3 mL of KOH was added into the provided compartment of the test flask. Consequently, these reactors have been placed in a chamber kept at a temperature 28°C. In order to verify that endogenous respiration is similar in different reactors, the CO_2 was once monitored by means titrating the KOH solution with HCl. Another importance of this preliminary phase is to obtain oxidation of excess organic matter, in order to start the test with a lower endogenous respiration. This phase is protracted for a week. A first titration was performed for both sediments: Greece and Italy. Based on the results of both titrations: it could be concluded that a preliminary phase of 7 days was sufficient. In total, 15 test flasks (each with a 300 mL volume) were prepared. Three flasks for each specimen and 3 control flasks. Preferably, the specimens were circular with weights of approximately 20 mg. According to the latter criteria, all samples were cut into circles with varying areas (about 6/8 cm^2 PBSe, PBSeT and LDPE and 2 cm^2 PHB that had a greater thickness). Subsequently, the test materials were placed at the top of the sediment, at the intermediary phase between sediment and seawater. Figure 34 shows the use of a mosquito net (circular shape) to prevent floating of the test materials.

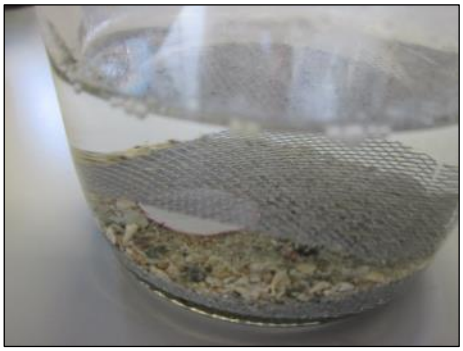


Figure 34. Visual presentation of the use of a mosquito net to prevent floating

The period of time between the carbon dioxide analysis by means of titrations were variable. Titrations were performed with the recommended frequency of every 3 days the first 2 weeks and every 1 to 3 weeks thereafter. With regard to the KOH solution: 3 mL of KOH solution with a molarity of 0.5 N was used. The titration was performed with 0.1 N HCl. Before filling the KOH container, the reactors remained open for about 15 minutes allowing the air in the reactors to refresh. Figure 35 shows the evolution of the biodegradation of the different samples in the sediment/seawater interface from Greece. The test is stopped after 181 days. Results are not reliable. The difference in biodegradation pattern between the samples is logical (PHB > PBSe and PBSeT > LDPE), but the biodegradation percentages are strongly overestimated and completely unrealistic (PHB copolymer: ± 250% after 181 days and LDPE: ± 100% after 181 days).

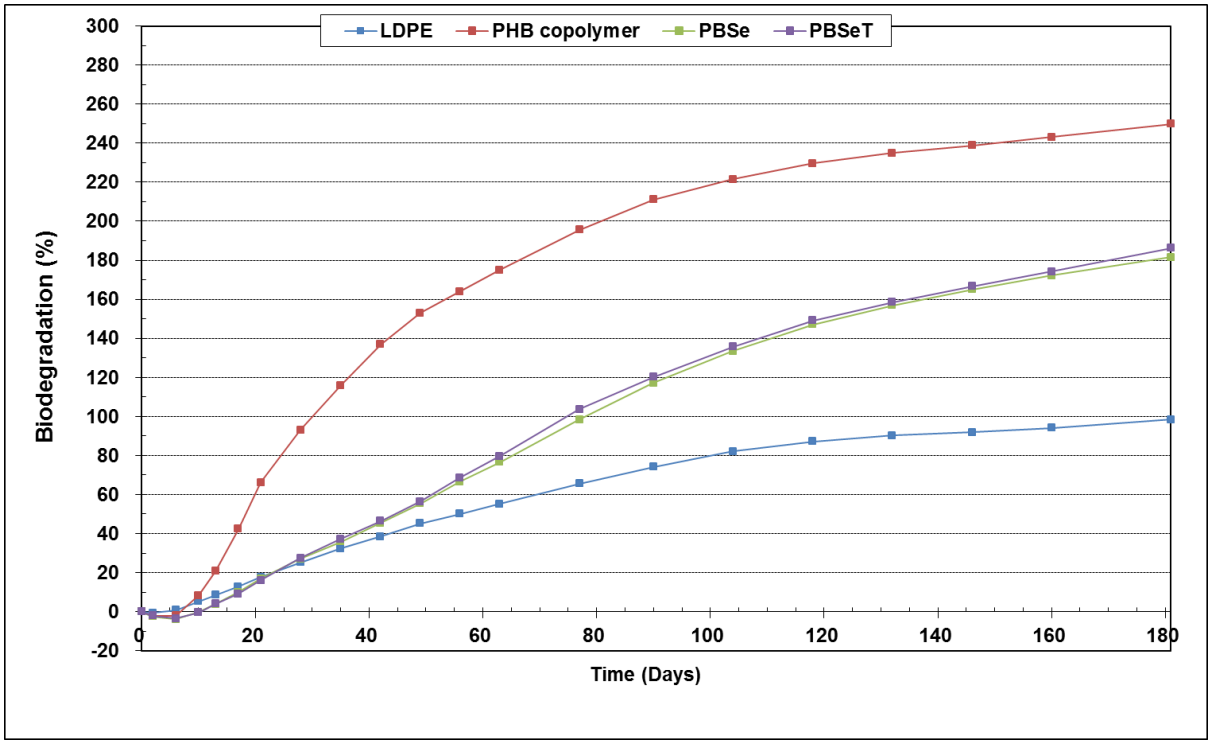


Figure 35. Evolution of biodegradation in sediment/seawater interface (Greece)

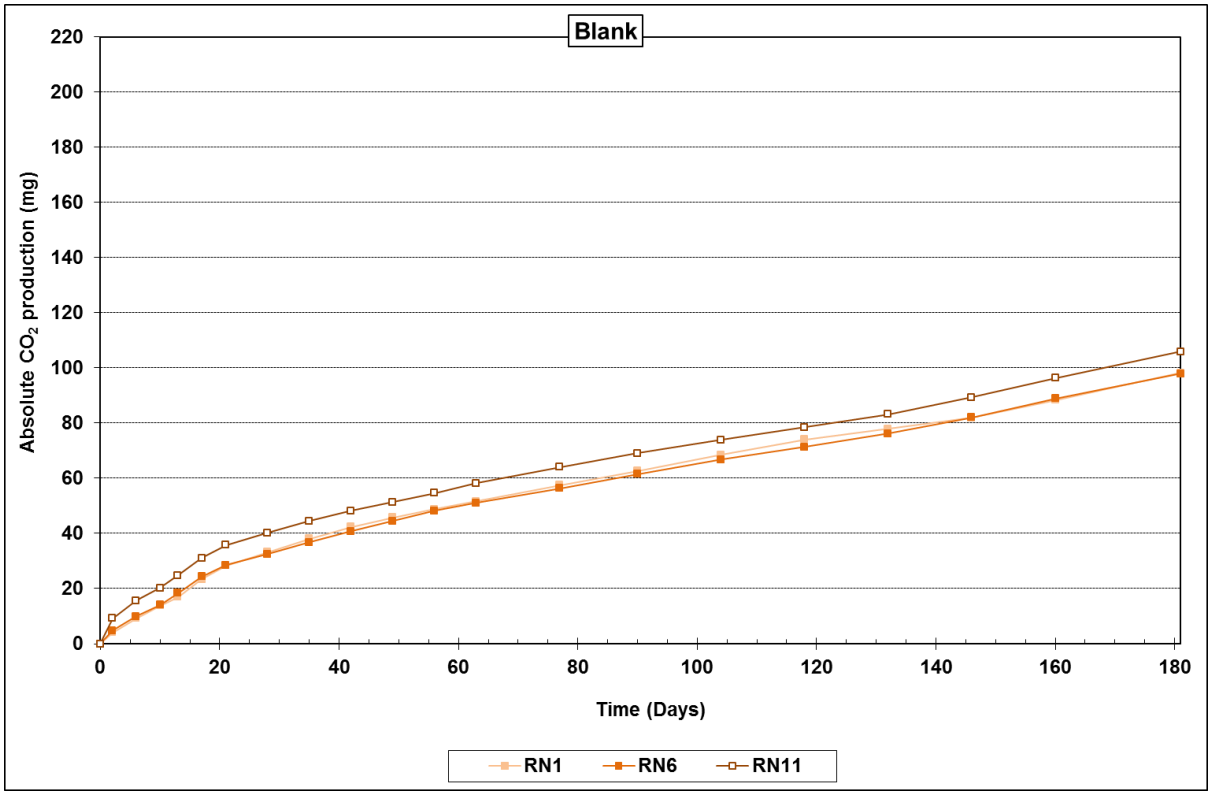


Figure 36. Total CO_2 production in blank reactors in sediment/seawater interface (Greece)

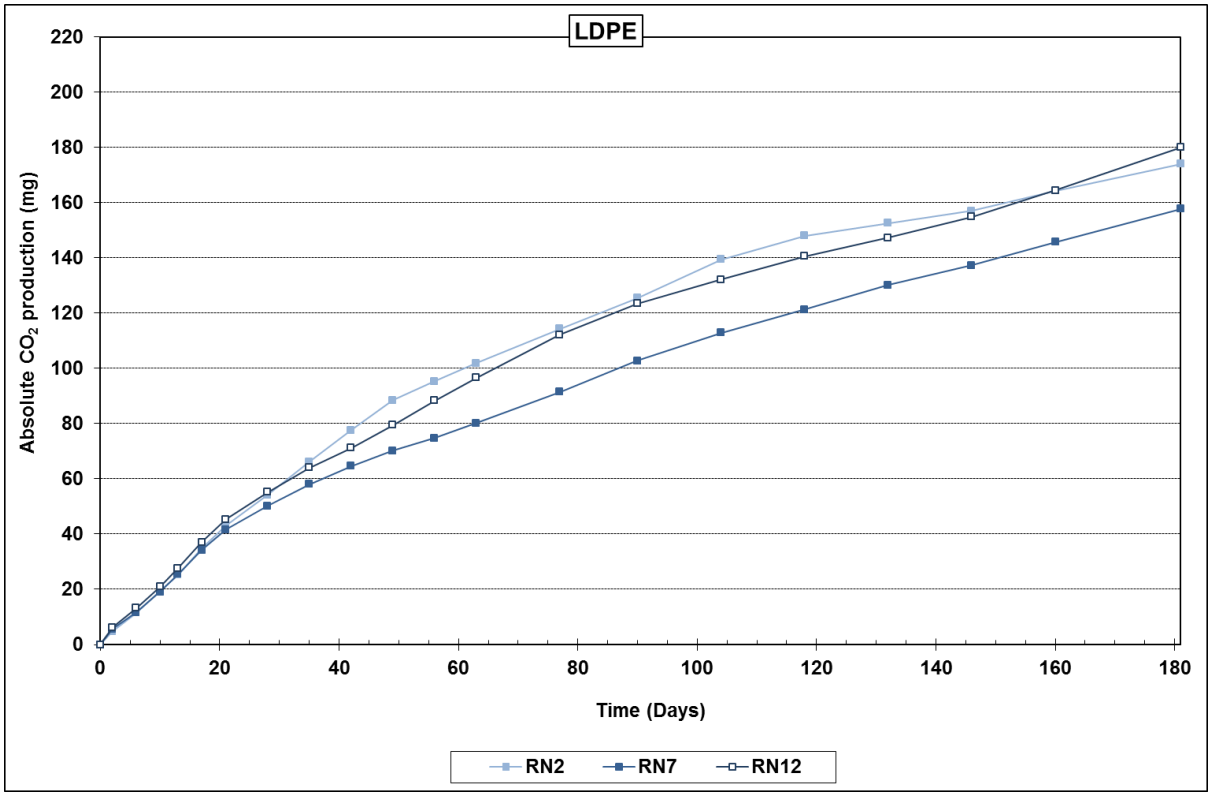


Figure 37. Total CO_2 production in LDPE reactors in sediment/seawater interface (Greece)

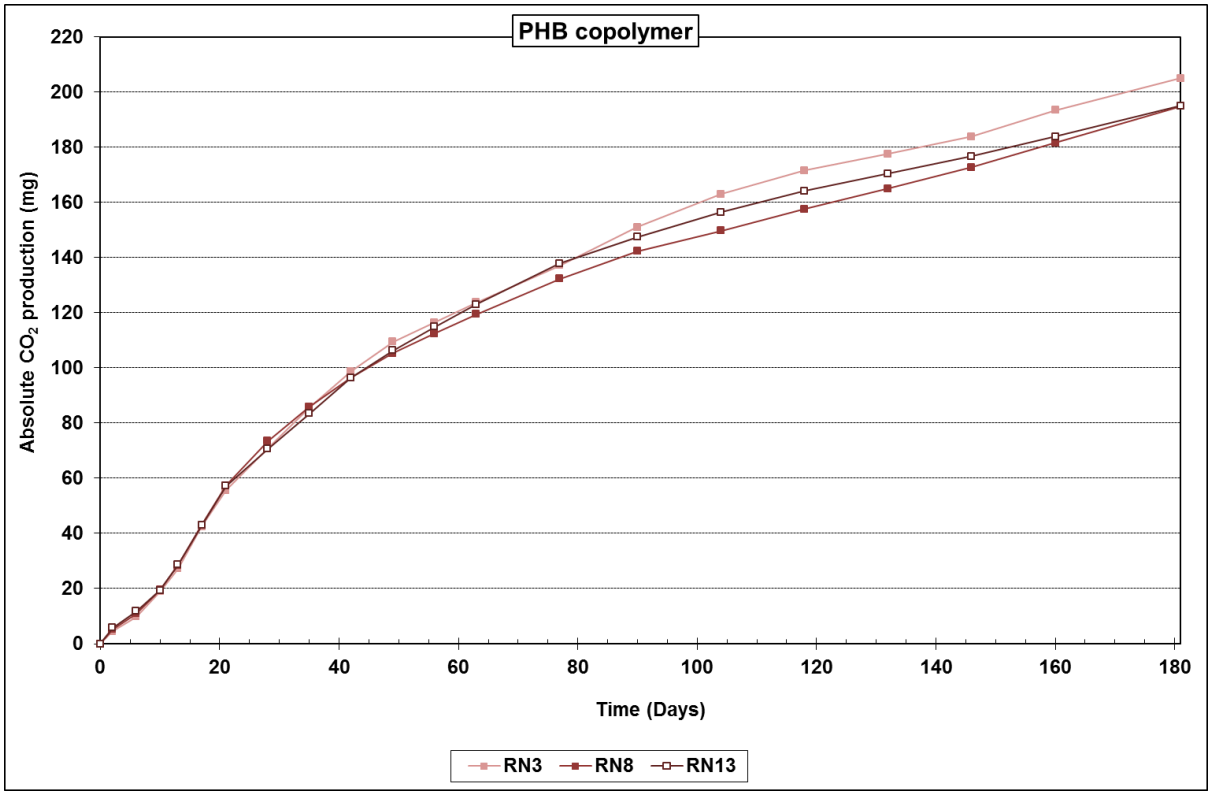


Figure 38. Total CO_2 production in PHB reactors in sediment/seawater interface (Greece)

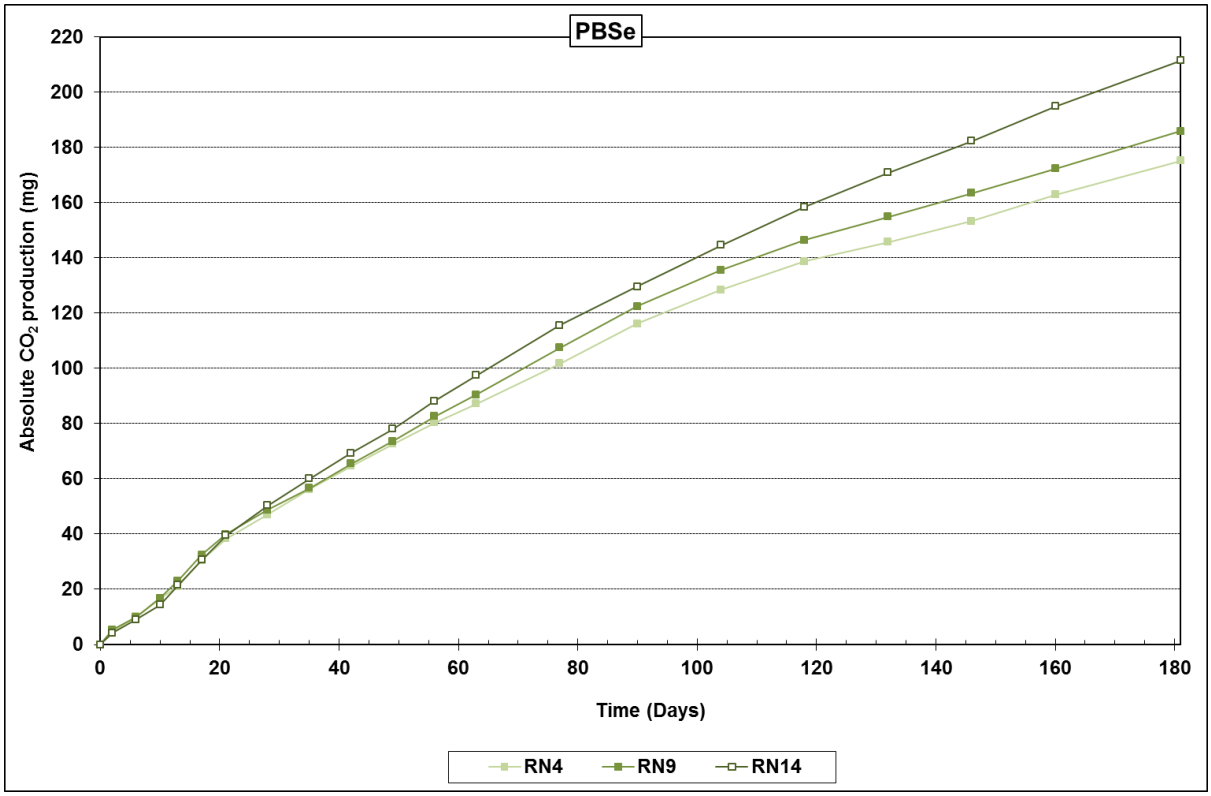


Figure 39. Total CO_2 production in PBSe reactors in sediment/seawater interface (Greece)

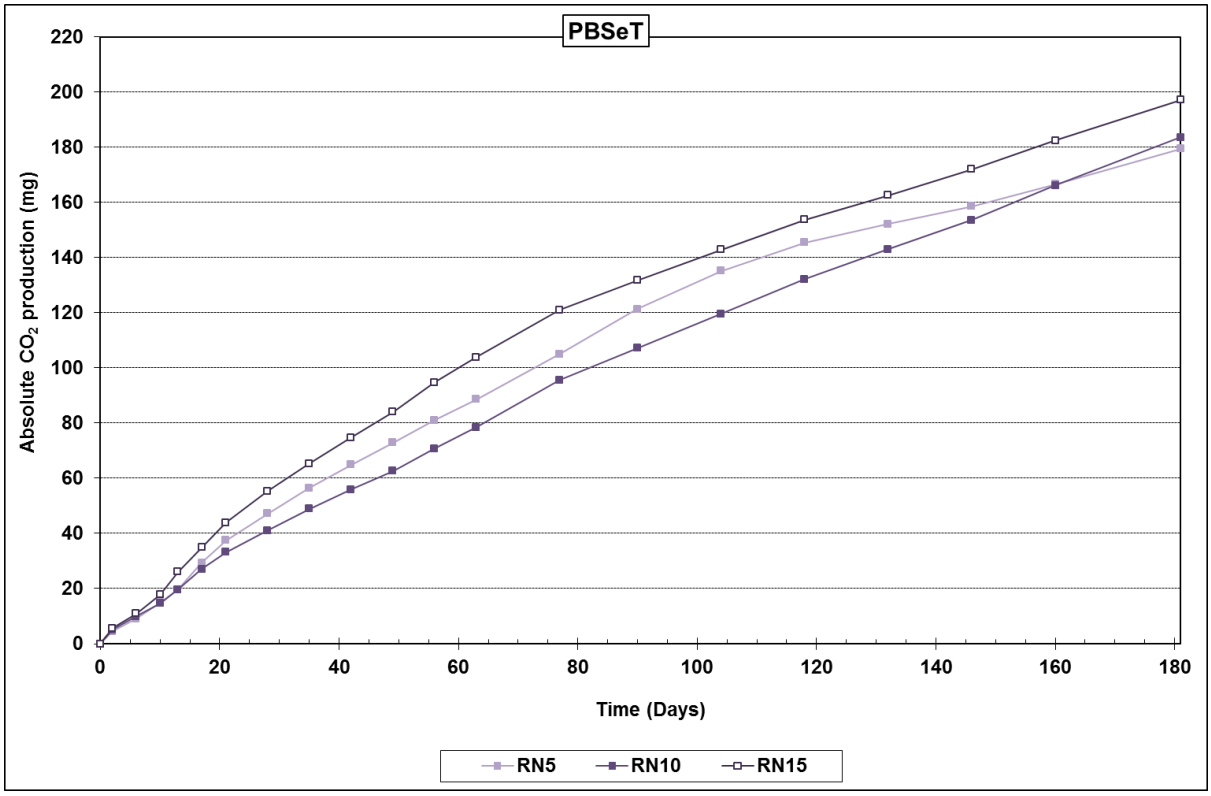


Figure 40. Total CO_2 production in PBSeT reactors in sediment/seawater interface (Greece)

At the end of the test (= after 181 days) the remaining test material was manually retrieved, dried and weighted. Table 15 gives an overview of the weights of the samples at start and at the end of the test and the disintegration percentage. An overview of the retrieved sample is given in Figure 41. Comparing numerical data with the visual observations, only the results of LDPE and PHB seem to be reliable. LDPE does not disintegrate and the sample is completely retrieved from the reactors, whereas for PHB only a low amount of sample was retrieved from one of the reactors. For PBSe and PBSeT the measurement of the disintegration is difficult due to contamination of the sample. Not all grains of sand can be removed from the sample (particularly if it is folded) and this generates an overestimation of the final weight of sample, that often is higher than the weight at the beginning of the test.

Table 15. Description and disintegration percentage of retrieved test materials after 181 days in seawater/sediment interface (Greece)

Test item	Weight sample at start (mg)	Weight sample at end (mg)	Disintegration (%)
LDPE	RN2 = 23.2 RN7 = 22.4 RN12 = 22.7	RN2 = 25.3 RN7 = 24.6 RN12 = 23.8	RN2 = 0** RN7 = 0** RN12 = 0**
PHB	RN3 = 22.2 RN8 = 21.8 RN13 = 22.9	RN3 = n.r.* RN8 = n.r.* RN13 = 3.4	RN3 = 100 RN8 = 100 RN13 = 85.2
PBSe	RN4 = 23.1 RN9 = 19.7 RN14 = 20.2	RN4 = 8.9 RN9 = 25.3 RN14 = 23.4	RN4 = 61.5 RN9 ** RN14 **
PBSeT	RN5 = 19.7 RN10 = 19.8 RN15 = 18.8	RN5 = 25.6 RN10 = 25.7 RN15 = 30.5	RN5 ** RN10 ** RN15 **

* n.r. = sample not recoverable

** weight of the retrieved sample at the end of the test is higher than the weight of the sample at start

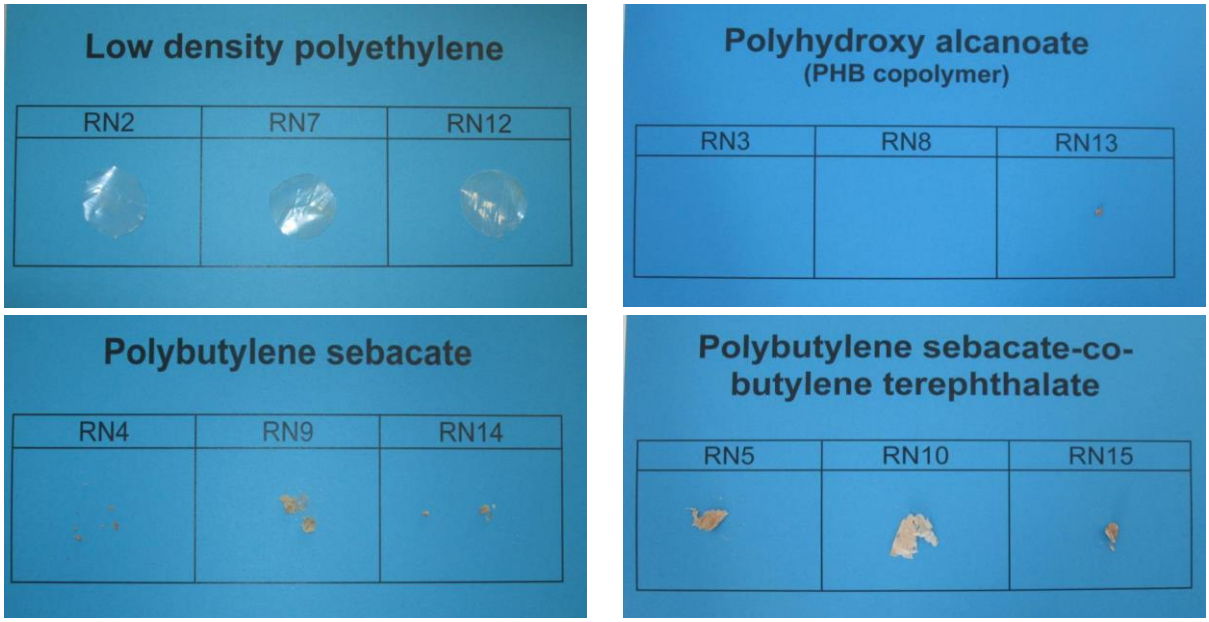


Figure 41. pictures of the residual test materials retrieved after the experiments

Figure 42 shows the evolution of the biodegradation of the different samples in sediment/seawater interface from Italy. Results are not reliable. Biodegradation of PHB has clearly reached a too high value (237.6% at the end of the test = after 182 days). The biodegradation of PBSe and PBSeT has proceeded at a comparable rate and slightly faster

when compared to negative reference material LDPE (difference is less obvious when compared to the inoculum from Greece). For negative reference material LDPE a biodegradation value of 94.5% was obtained at the end of the test. This indicates that the biodegradation is significantly overestimated and should be corrected by subtracting CO_2 production in the reactors with LDPE.

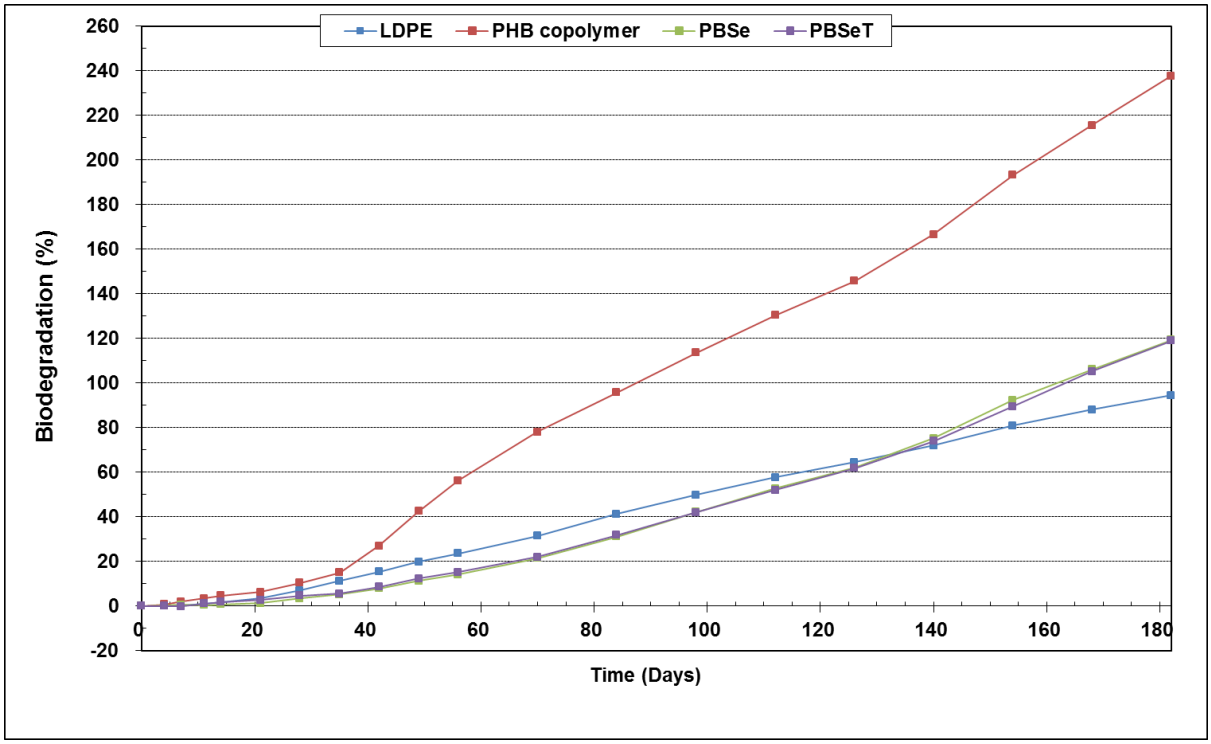


Figure 42. Evolution of biodegradation in sediment/seawater interface (Italy)

At the end of the test (after 182 days) the remaining test material was manually retrieved, dried and weighted. Table 16 gives an overview of the weights of the samples at the beginning and at the end of the test, an evaluation of the disintegration is also included. As for the results in the Greece samples, comparing numerical data with the visual observations, only the results of LDPE and PHB seem to be reliable. Effectively, LDPE does not disintegrate and the sample is completely retrieved from the reactors (see Figure 48), whereas for PHB the sample was not retrieved from the reactors. Based on the disintegration percentages of PBSe, the disintegration has started in one of the reactors (RN14) and not for the others, but this is in contrast with the visual observations. The sample from RN14 is intact, whereas for the other reactors the disintegration has started, in particular in reactor RN4, as showed in Figure 48. Based on the disintegration percentage of PBSeT, the disintegration has started in one of the reactors (RN15), but this is in contrast with the visual observation (Figure 48 shows an intact piece). Again it can be concluded that not all grains of sand can be removed from the sample and this generates an overestimation of the final weight of sample, that often is higher than the weight at the beginning of the test.

The evolution of the total CO_2 production of the individual replicates is given in Figure 43 up to Figure 47.

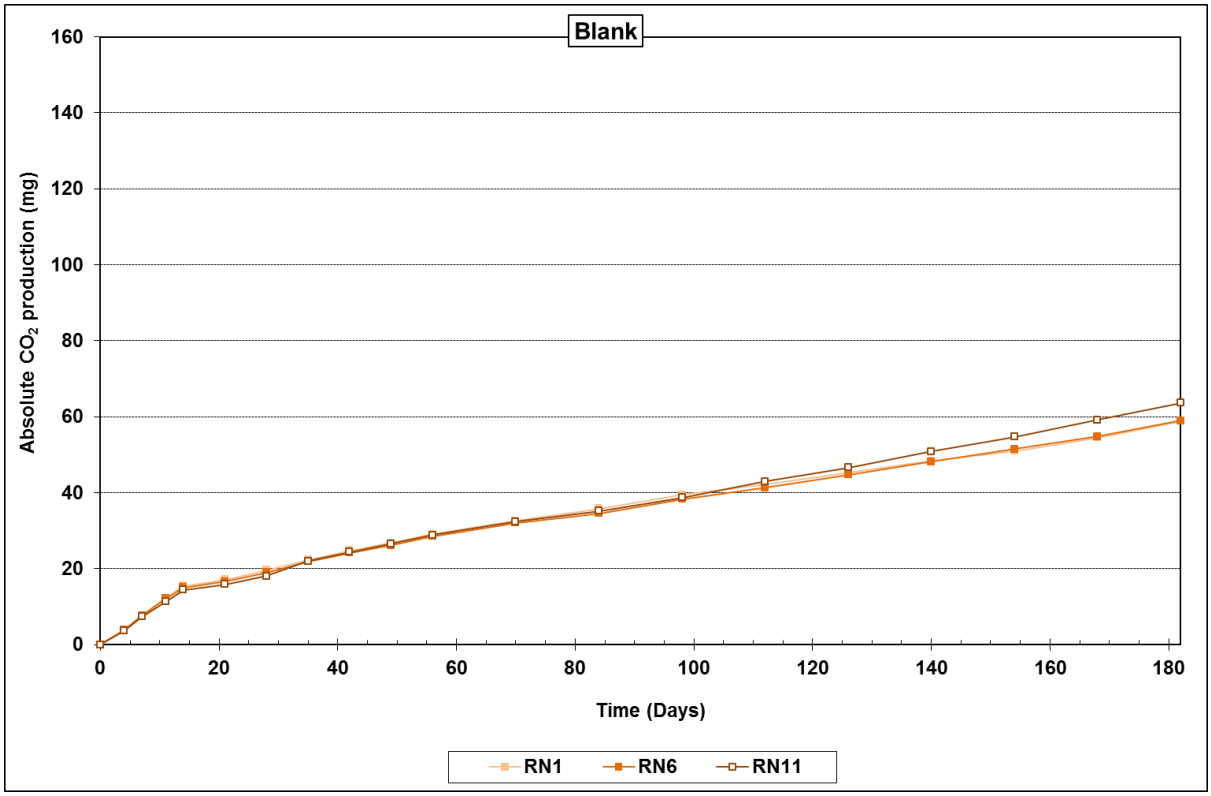


Figure 43. Total CO_2 production in blank reactors in sediment/seawater interface (Italy)

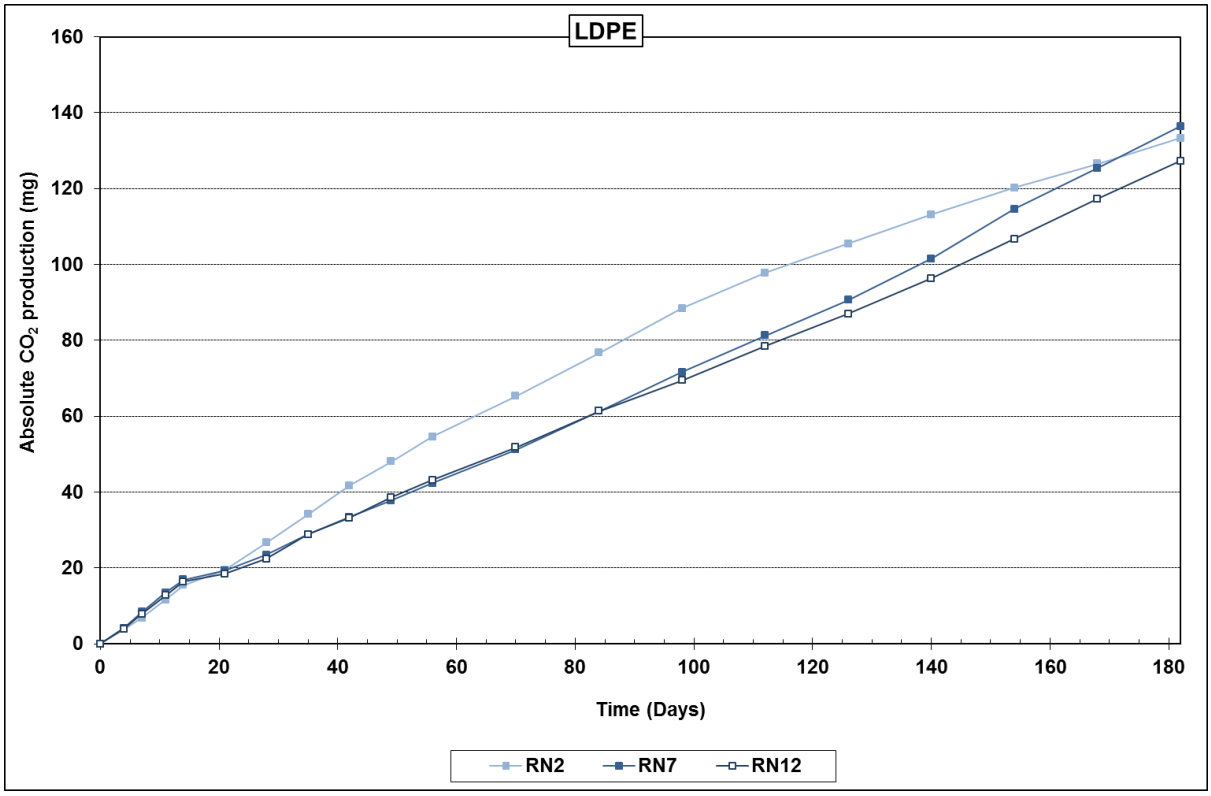


Figure 44. Total CO_2 production in LDPE reactors in sediment/seawater interface (Italy)

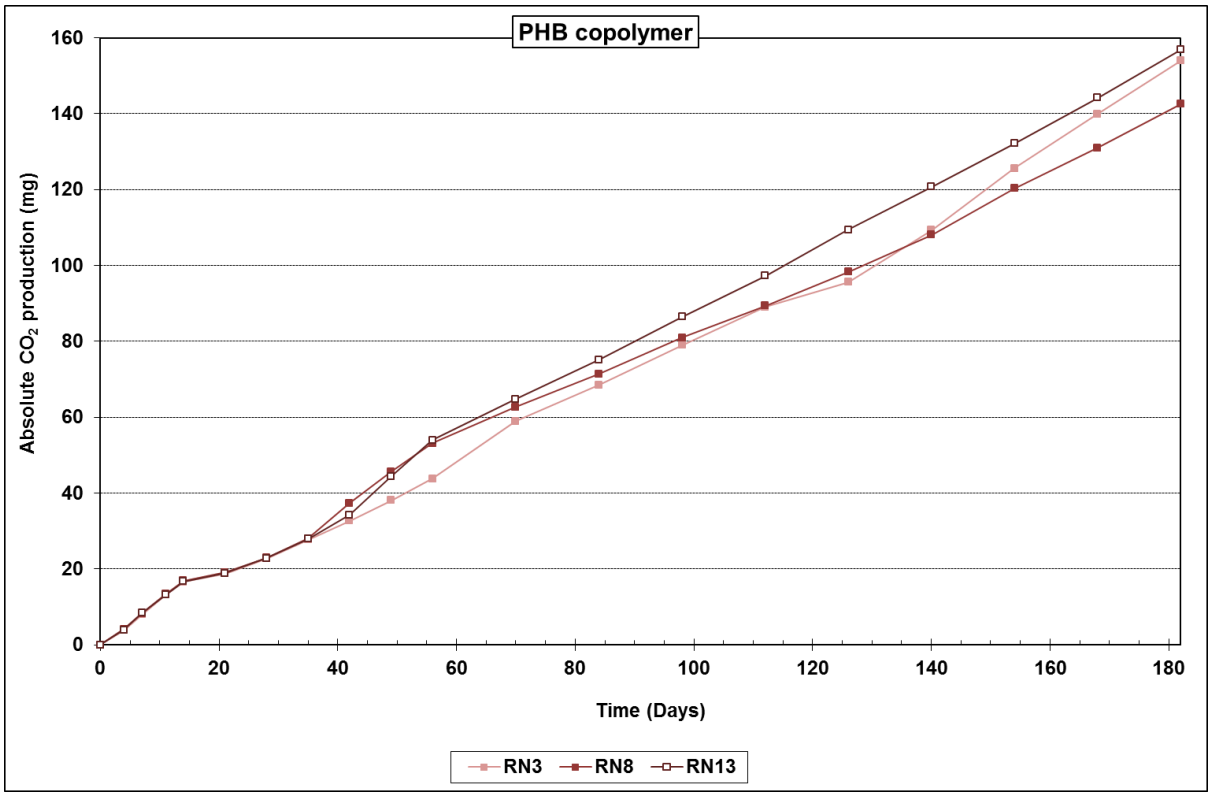


Figure 45. Total CO_2 production in PHB reactors in sediment/seawater interface (Italy)

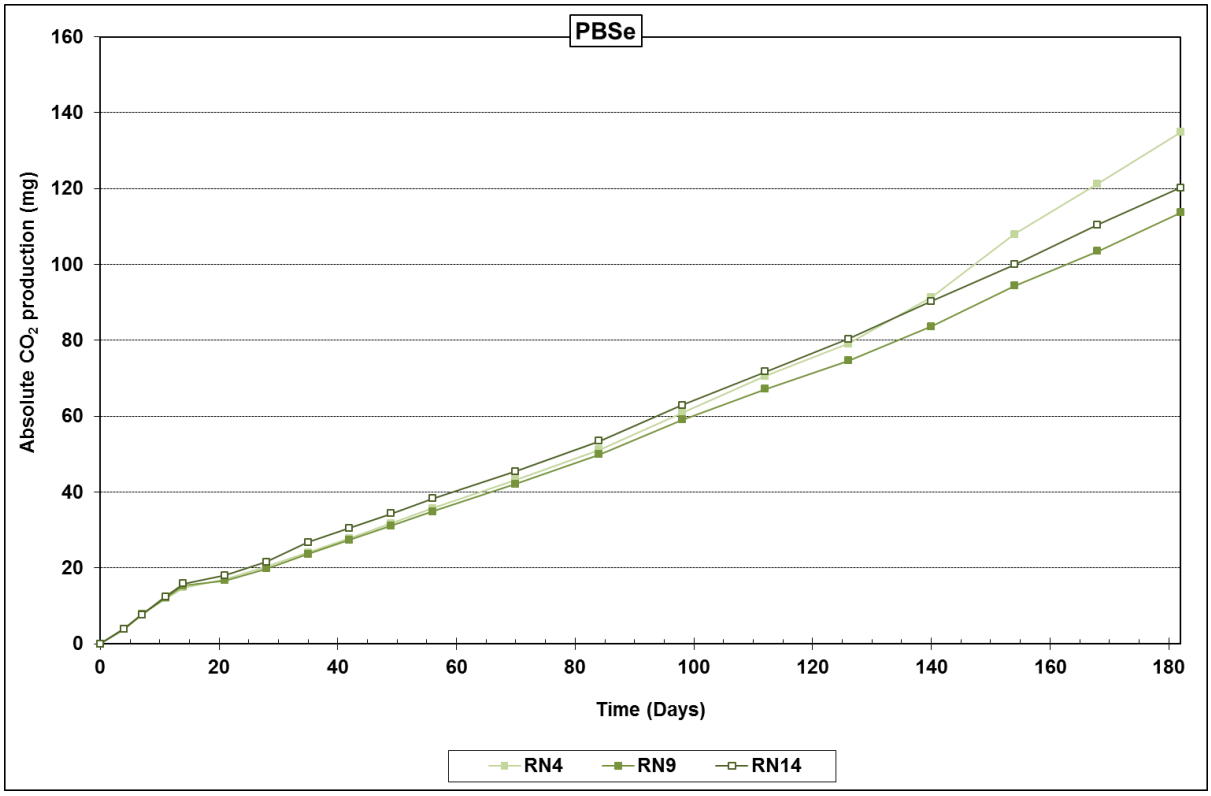


Figure 46. Total CO_2 production in PBSe reactors in sediment/seawater interface (Italy)

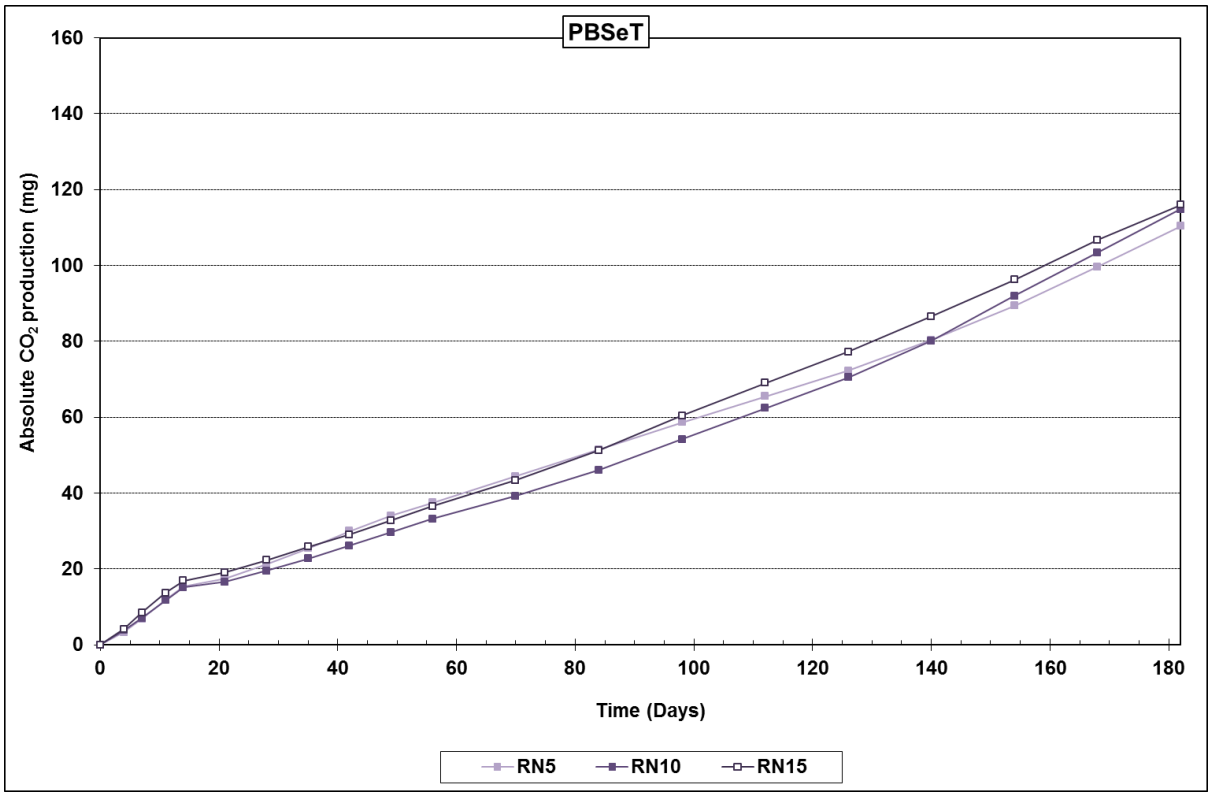


Figure 47. Total CO_2 production in PBSeT reactors in sediment/seawater interface (Italy)

At the end of the test (= after 182 days) the remaining test material was manually retrieved, dried and weighted. Table 16 gives an overview of the weights of the samples at the beginning and at the end of the test, an evaluation of the disintegration is also included. An overview of the retrieved samples is given in Figure 48. As for the results in the Greece samples, comparing numerical data with the visual observations, only the results of LDPE and PHB seem to be reliable. Effectively, LDPE doesn't disintegrate and the sample is completely retrieved from the reactors, whereas for PHB the sample was not retrieved from the reactors. Based on the disintegration percentages of PBSe, the disintegration has started in one of the reactors (RN14) and not for the others, but this is in contrast with the visual observation. The sample from RN14 is intact, whereas for the other reactors the disintegration has started (in particular in reactor RN4). Based on the disintegration percentage of PBSeT, the disintegration has started in one of the reactors (RN15), but this is in contrast with the visual observation (Figure 48 shows an intact piece). Again it can be concluded that not all grains of sand can be removed from the sample and this generates an overestimation of the final weight of sample, that often is higher than the weight at the beginning of the test.

Table 16. Description and disintegration percentage of retrieved test materials after 182 days in seawater/sediment interface (Italy)

Test item	Weight sample at start (mg)	Weight sample at end (mg)	Disintegration (%)
LDPE	RN2 = 23.6 RN7 = 24.6 RN12 = 25.1	RN2 = 25.7 RN7 = 27.0 RN12 = 26.3	RN2 = 0** RN7 = 0** RN12 = 0**
PHB	RN3 = 21.7 RN8 = 21.9 RN13 = 21.7	RN3 = n.r.* RN8 = n.r.* RN13 = n.r.*	RN3 = 100 RN8 = 100 RN13 = 100
PBSe	RN4 = 21.8 RN9 = 21.8 RN14 = 22.1	RN4 = 22.5 RN9 = 27.4 RN14 = 21.1	RN4 ** RN9 ** RN14 = 4.52
PBSeT	RN5 = 19.7 RN10 = 17.4 RN15 = 19.3	RN5 = 25.1 RN10 = 17.6 RN15 = 16.5	RN5 ** RN10 ** RN15 =14.51

* n.r. sample not recoverable

** the weight of the retrieved sample at the end of the test is higher than the weight of the sample at start

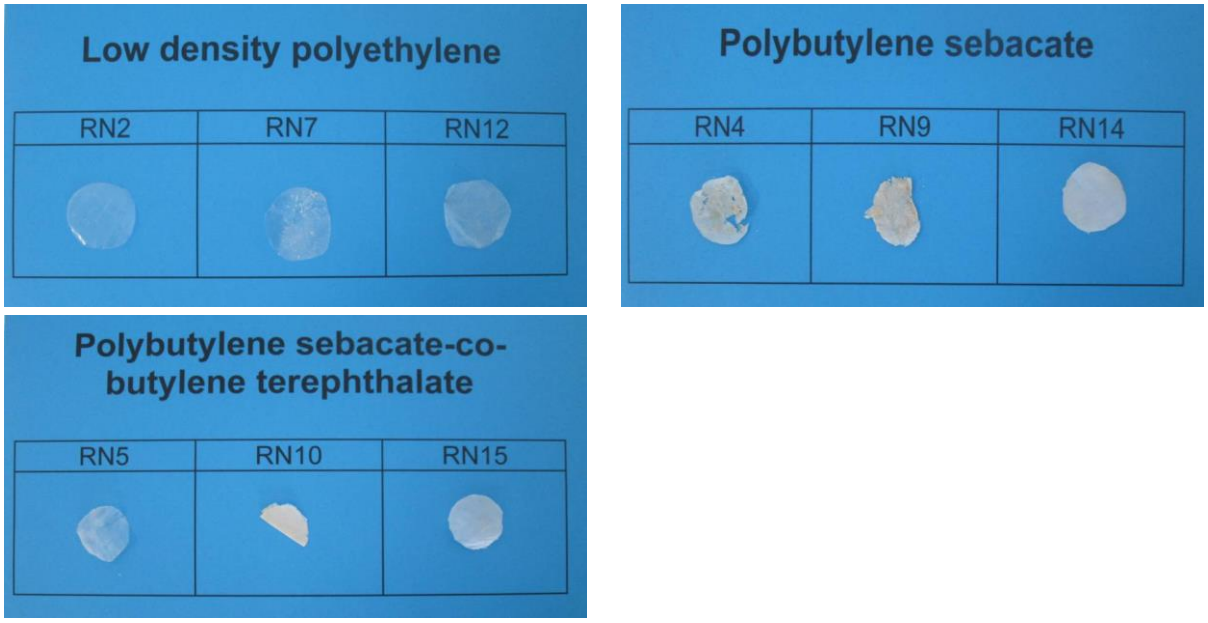


Figure 48. Overview of retrieved pieces of test materials in sediment/seawater interface (Italy) (sample in RN10 was folded during the drying process)

The Biodegradation in this interface system (sediment - seawater) is clearly overestimated. The executed tests are not reliable.

Possible solutions:

- Perforation of the sample: a comparative test between perforated and non-perforated sample (PHB and LDPE) was started on Nov-13-14. The test was stopped after 58 days due to high variation between the blank reactors -> no conclusions were made. No additional testing will be performed as Novamont already investigated this.
- Addition of inert material on the bottom of the blank reactors? (Disadvantage: when the test material will degrade the plastic layer will disappear during the test, while this will not be the case when the inert material is used)
- Increase the duration of the degradation of the organic matter in the sediment during the preliminary phase
- Reduce the background activity of the sediment by diluting the fresh sediment with sediment without organic matter

5.2.3 Biodegradation in Pelagic zone

An uniform sample of seawater was taken from the sea in Greece and Italy, respectively, by the Agricultural University of Athens and by the Hydra Institute for Marine Science. The seawaters were stored at approximately 4°C. There was no need to remove plant material, sea shell, pieces of driftwood, or other large pieces of material because of the purity of the 2 seawaters. No preliminary phase was performed. Before the start of the tests, the seawaters were analyzed the results are summarized in Table 3 and 4. KH_2PO_4 and NH_4Cl were added to the seawaters. They serve as nutrients for the micro-organisms, which

stand in for the biodegradation of the specimens. It was decided to add 0.05 g NH_4Cl and 0.1 g KH_2PO_4 per liter seawater. Accidentally, K_2HPO_4 (instead of KH_2PO_4) was added to the flasks of the test with the seawater of Greece. That means that the seawater of Greece contains more K and less PO_4 (respectively +0.0162 g/L and -0.0153 g/L) compared with the seawater of Italy. The 2 tests were started simultaneously with the same test items as mentioned before (LDPE, PHB, PBSe and PBSeT). The test items were milled to powders. In total, 15 test flasks (each with a 500 mL volume) for each test were prepared. Three flasks for each specimen and 3 control flasks. Subsequently, the test flasks were filled with 250 mL seawater. Then 60 mg of each test material (milled form) was put in the seawater (not applied for the control reactors). Afterwards a NaOH-pellet was put in a specific designed object and a stir bar was put in the flasks. The flasks were put on magnetic stirrers and incubated at a temperature of 28 °C. The period of time between the carbon dioxide analysis by means of titrations were variable. Titrations were performed once a week, the first 3 weeks. And once every 2 weeks the weeks thereafter. Before placing a new NaOH-pellet, the reactors remained open for about 15 minutes allowing the air in the reactors to refresh. The pellets were dissolved in distilled water and titrated with an HCl solution of 0.1 N. At start, 2 NaOH-pellets were used per reactor. It seemed that this was not necessary because the biodegradation of the species in seawater wasn't as fast as expected. One pellet was sufficient enough to absorb the formed CO_2 during the process. Moreover a large volume of HCl solution was needed to titrate the 2 pellets. It was decided to use 1 pellet after the first titration.

Figure 49 shows the evolution of the biodegradation of the different samples in the seawater from Greece. The test was stopped after 180 days for LDPE and for PBSe, while the test was extended till 312 days for positive reference material PHB copolymer and PBSeT. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. Negligible biodegradation was observed for LDPE. At the end of the test (after 180 days), LDPE and PBSe have reached a biodegradation percentage of -3.9% ± 8.3% and 66.5% ± 8.1%, respectively. The biodegradation percentages of the other samples were 98.1% ± 39.2% for PHB copolymer and 72.8% ± 9.4% for PBSeT after 312 days. It must be noticed that one of the replicates of PHB copolymer is characterized by a significantly higher biodegradation when compared to the other 2 replicates (Figure 50). This replicate is not reliable and it would be more realistic to omit this replicate. Without taking into account this replicate a biodegradation percentage of 75.6% ± 5.7% is obtained for PHB copolymer after 312 days.

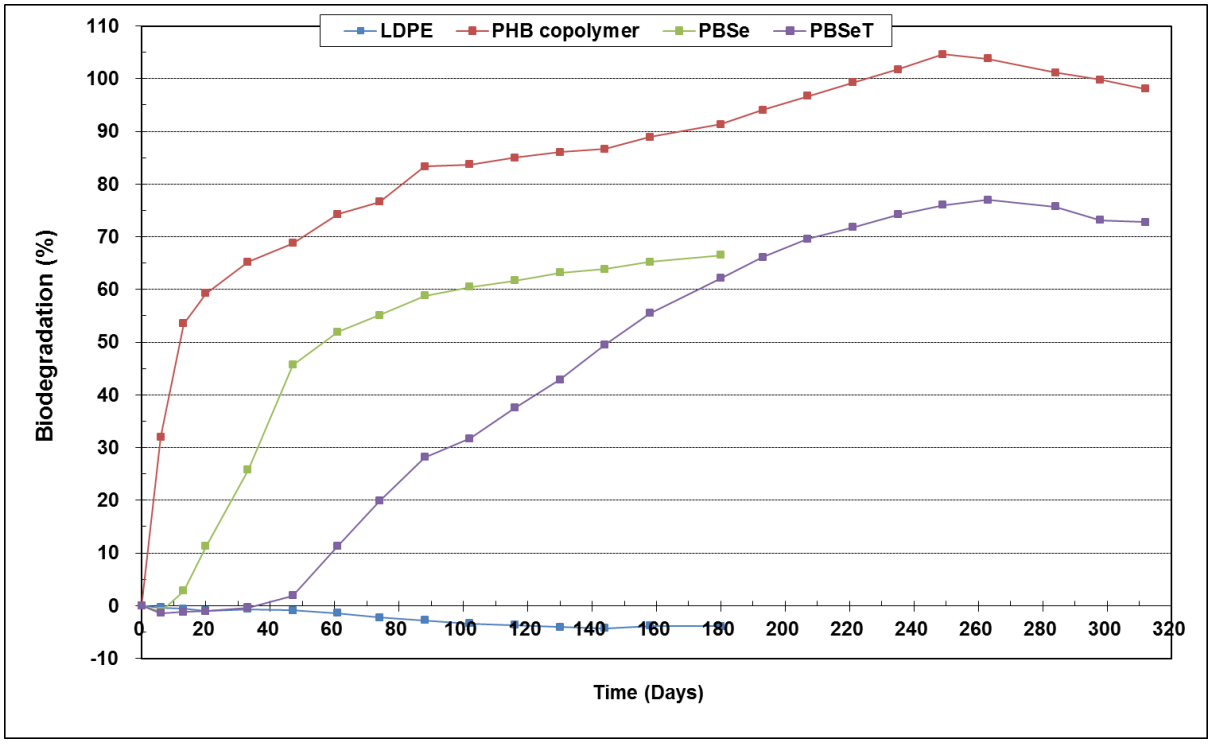


Figure 49. Evolution of biodegradation in seawater (Greece)

The evolution of the total CO_2 production of the individual replicates is given in Figure 51 up to Figure 55.

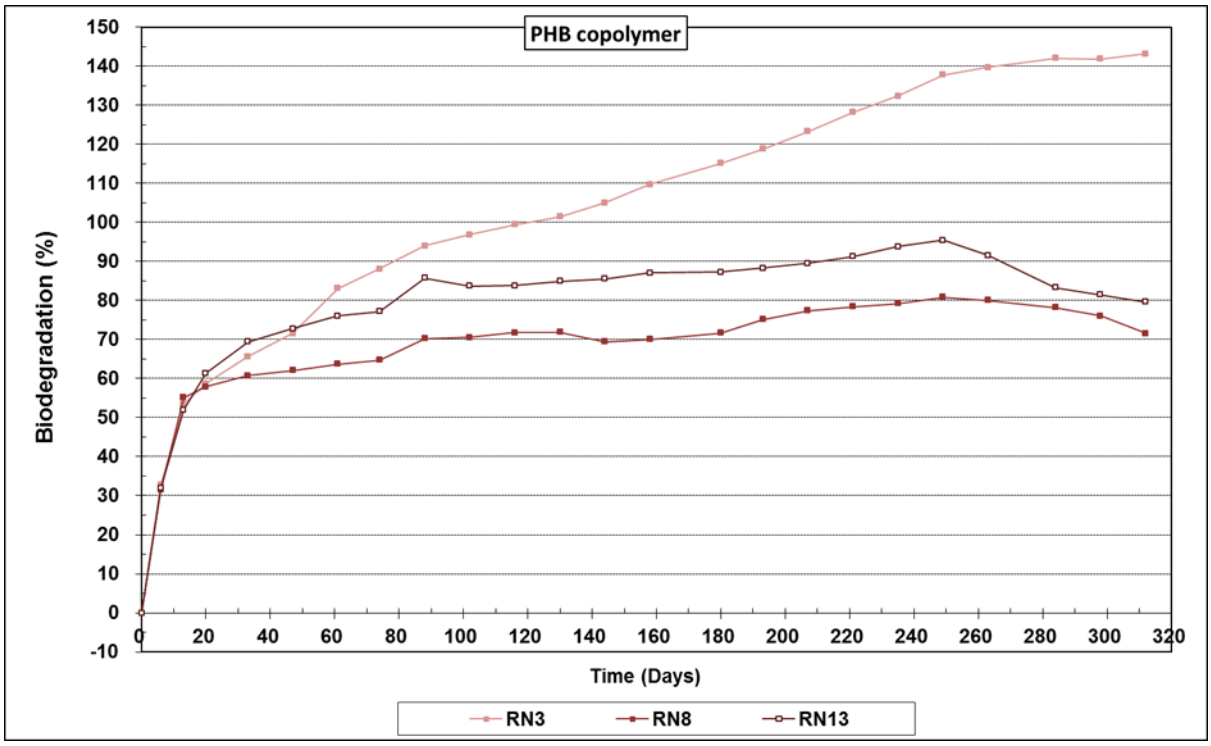


Figure 50. Evolution of biodegradation of the replicates of PHB copolymer in seawater (Greece)

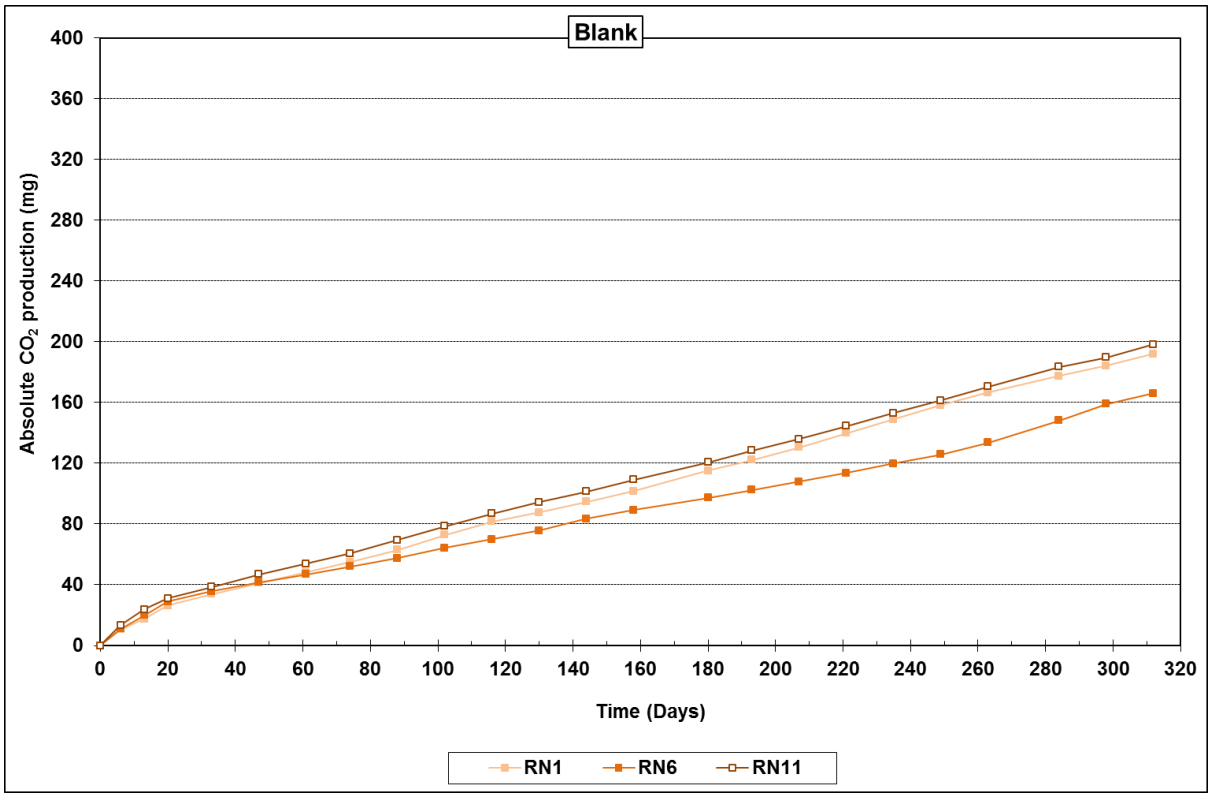


Figure 51. Total CO_2 production in blank reactors in seawater (Greece)

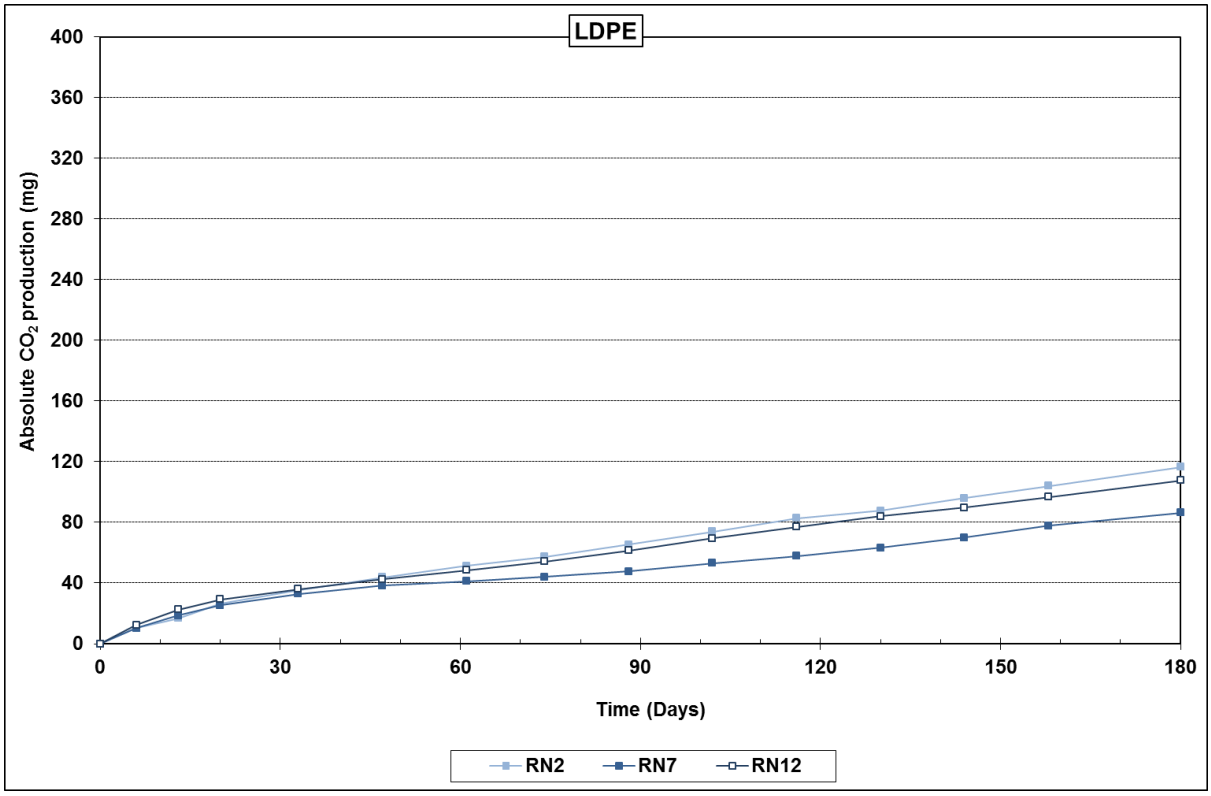


Figure 52. Total CO_2 production in LDPE reactors in seawater (Greece)

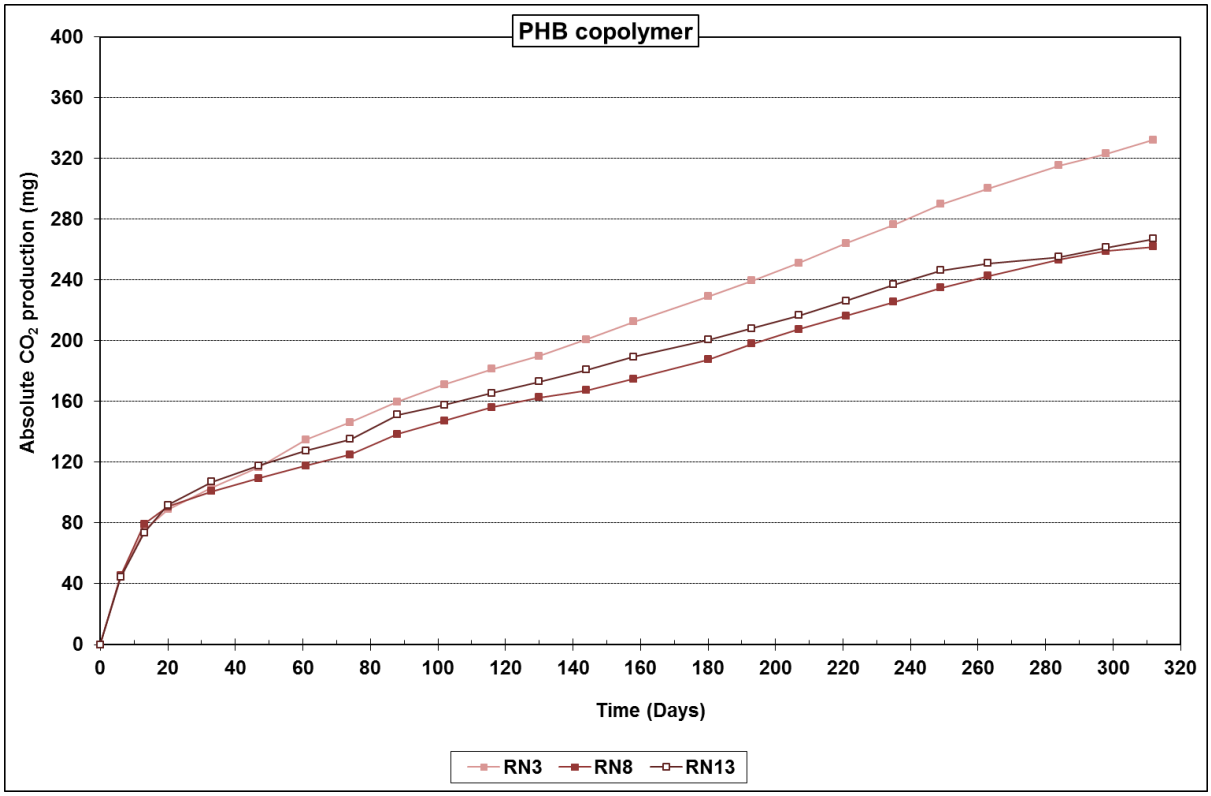


Figure 53. Total CO_2 production in PHB reactors in seawater (Greece)

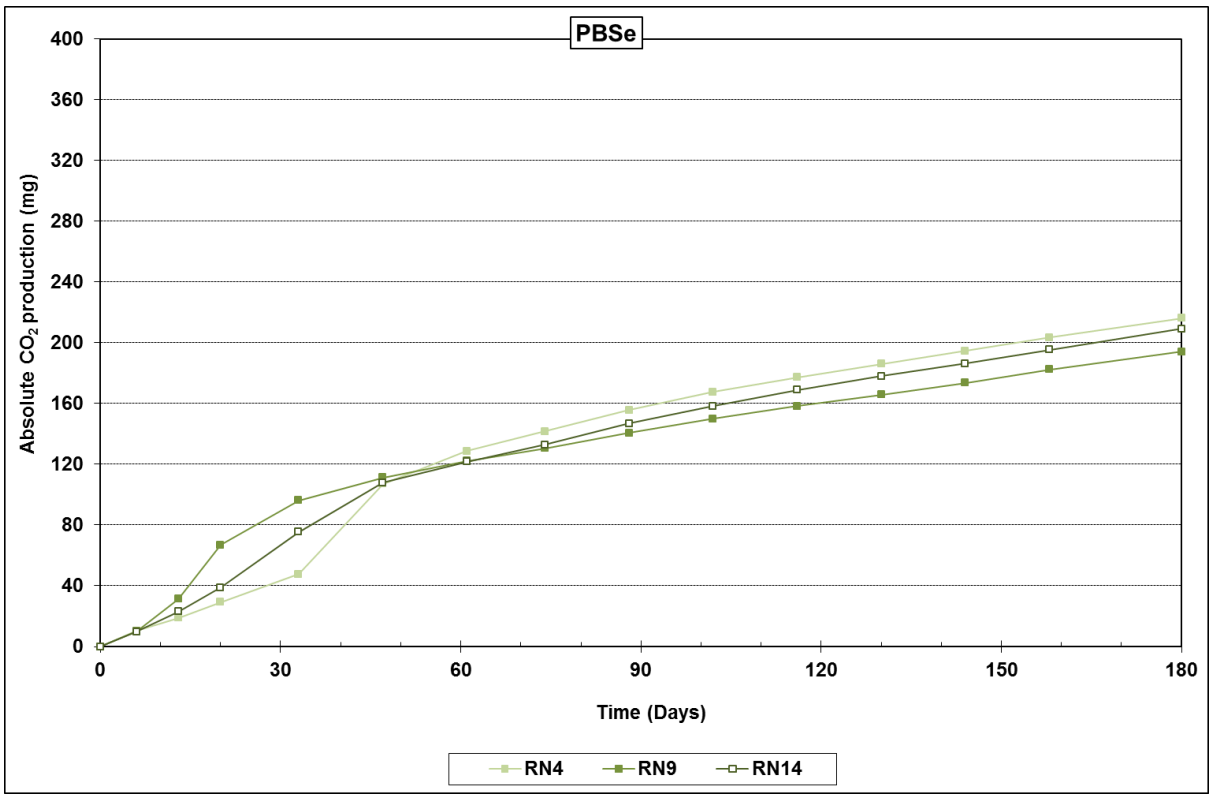


Figure 54. Total CO_2 production in PBSe reactors in seawater (Greece)

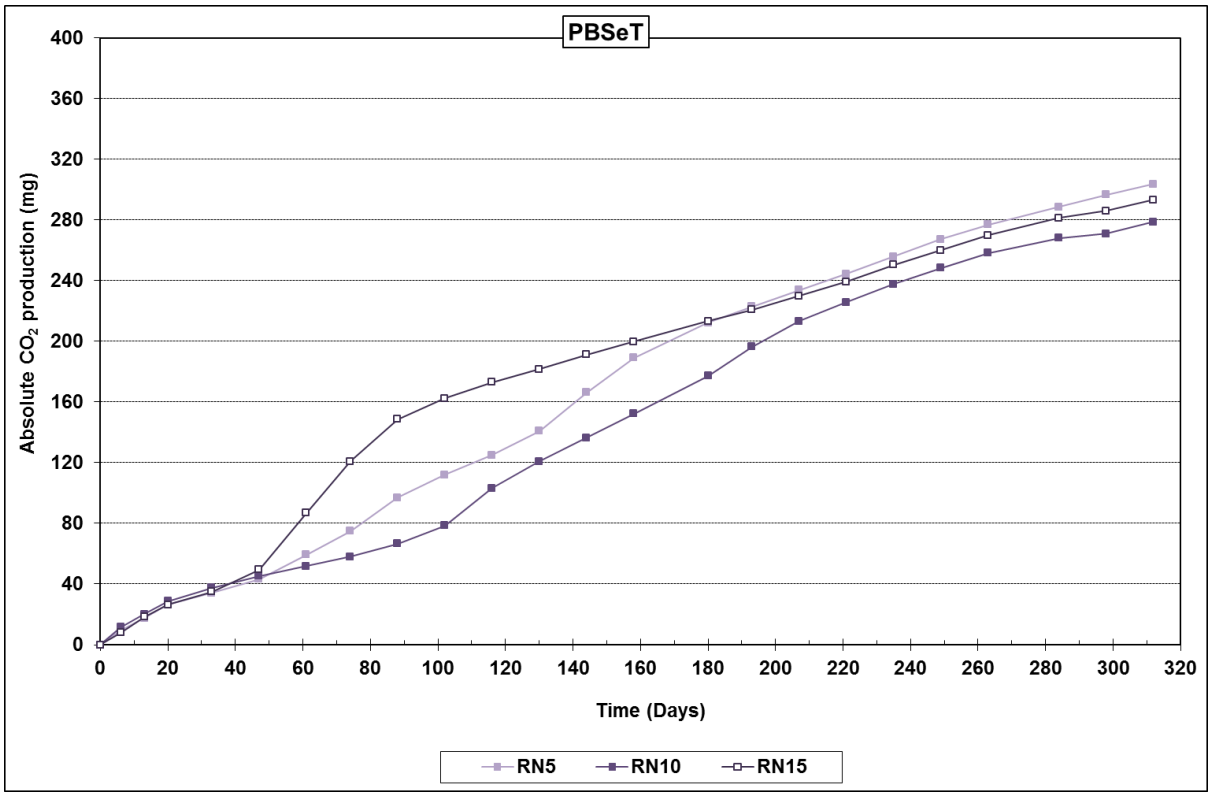


Figure 55. Total CO_2 production in PBSeT reactors in seawater (Greece)

Figure 56 shows the evolution of the biodegradation of the different samples in seawater from Italy. The test was stopped after 180 days for LDPE and for PBSe, while the test was extended till 312 days for positive reference material PHB copolymer and PBSeT. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. Negligible biodegradation was observed for LDPE. At the end of the test (= after 180 days), LDPE and PBSe have reached a biodegradation percentage of -2.2% ± 10.5% and 57.6% ± 7.0%, respectively. The biodegradation percentages of the other samples were 80.4% ± 47.6% for PHB copolymer and 47.8% ± 23.2% for PBSeT after 312 days (Figure 56). It must be noticed that one of the replicates of PHB copolymer is characterized by a significantly higher biodegradation when compared to the other 2 replicates. This replicate is not reliable and it would be more realistic to omit this replicate. Without taking into account this replicate a biodegradation percentage of 53.5% ± 13.7% is obtained for PHB copolymer after 312 days (Figure 57).

The evolution of the total CO_2 production of the individual replicates is given in Figure 58 up to Figure 62.

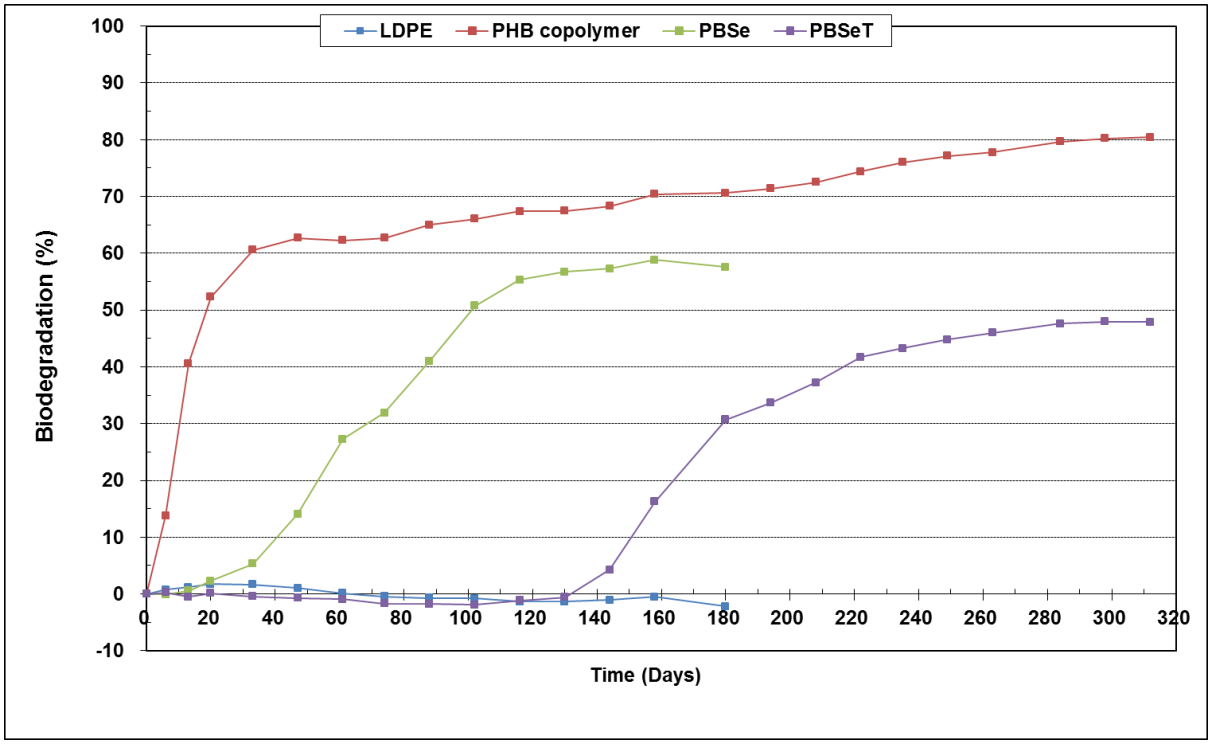


Figure 56. Evolution of biodegradation in seawater (Italy)

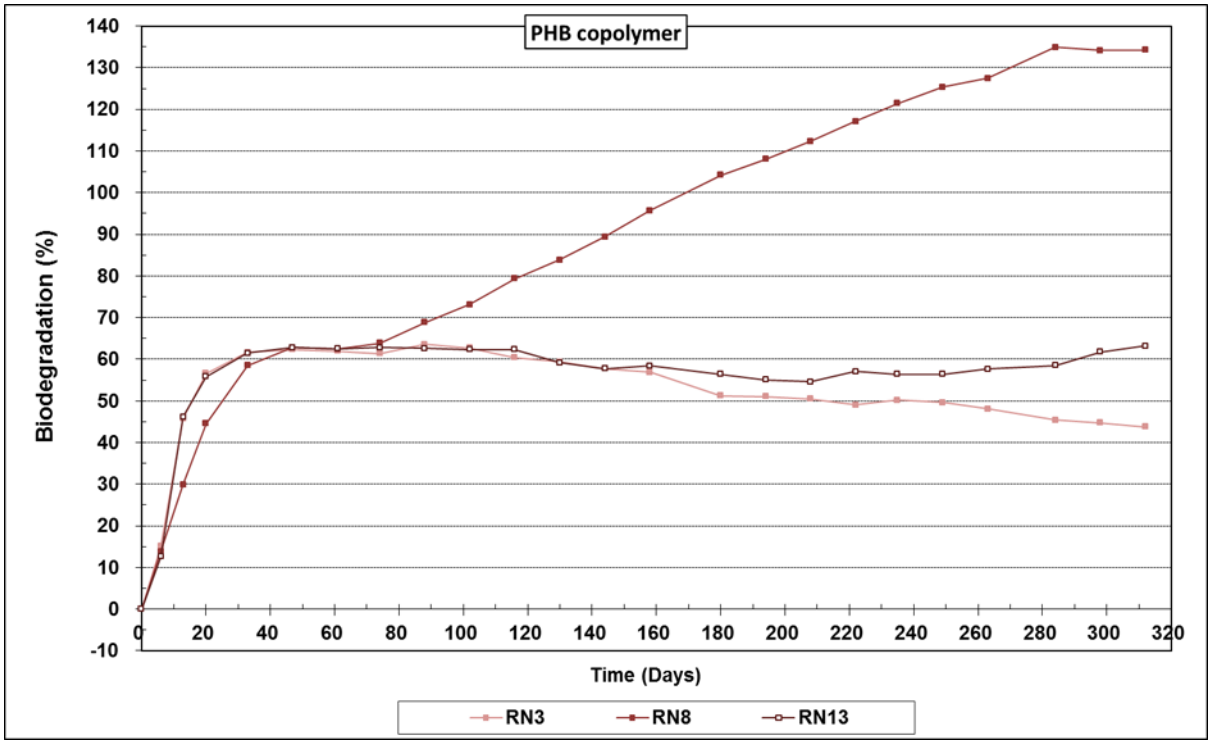


Figure 57. Evolution of biodegradation of the replicates of PHB copolymer in seawater (Italy)

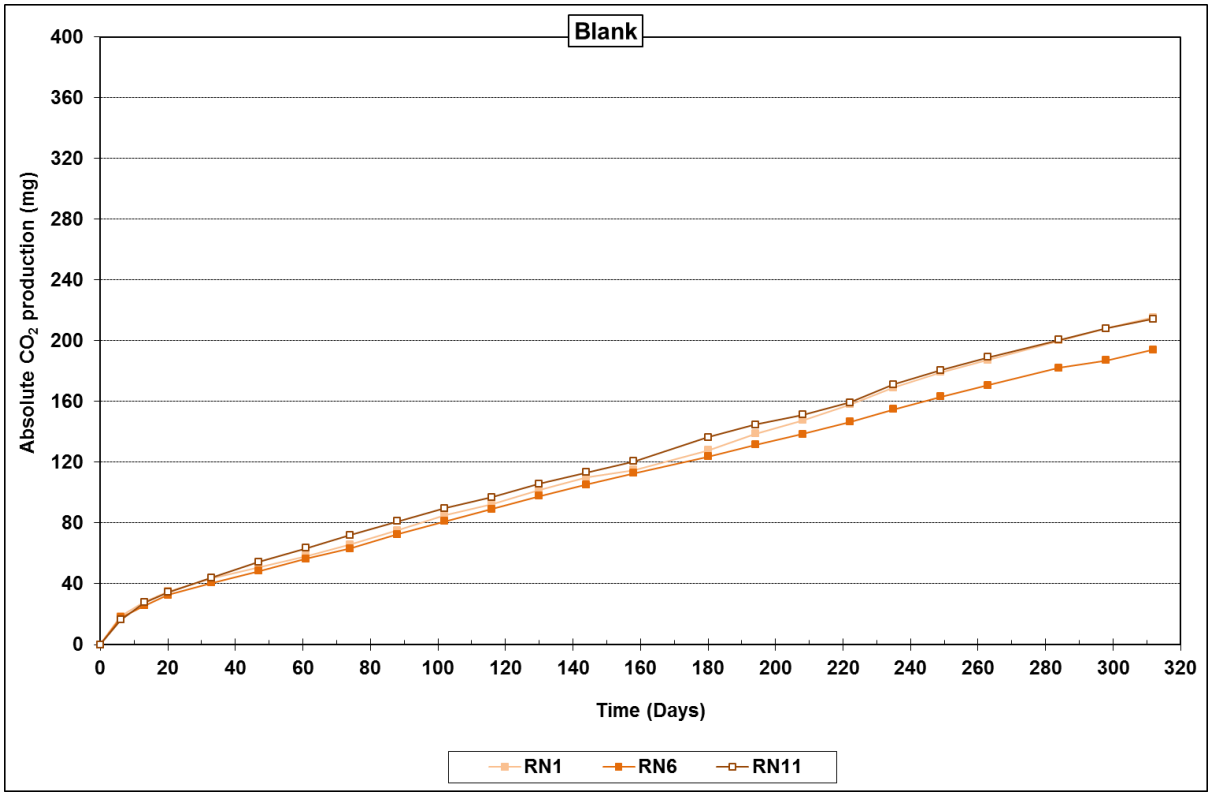


Figure 58. Total CO_2 production in blank reactors in seawater (Italy)

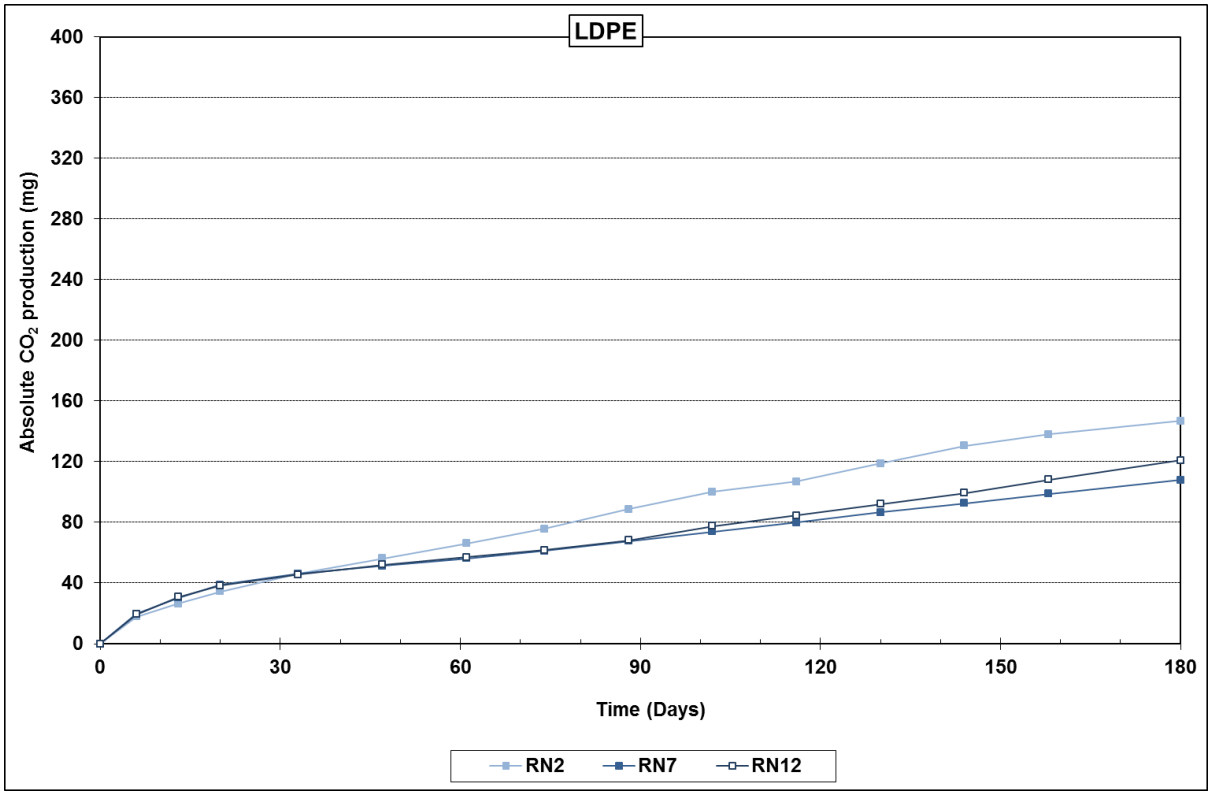


Figure 59. Total CO_2 production in LDPE reactors in seawater (Italy)

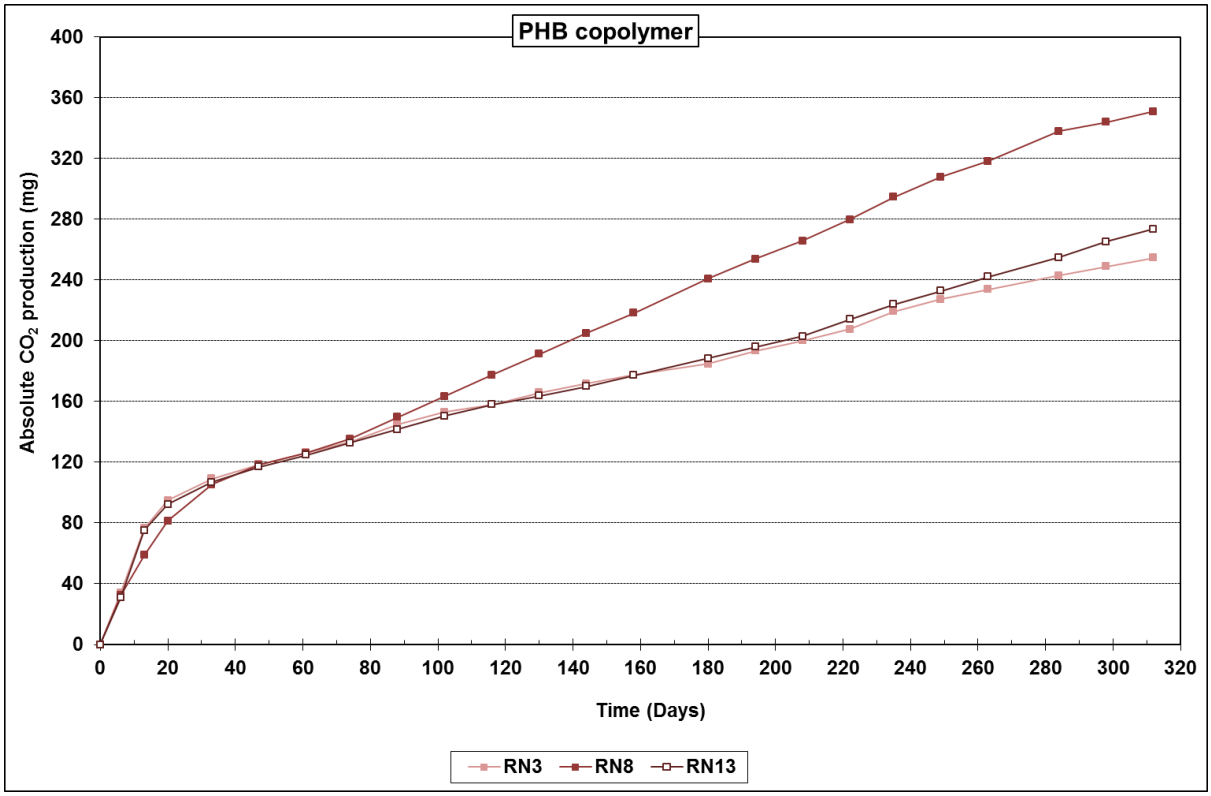


Figure 60. Total CO_2 production in PHB reactors in seawater (Italy)

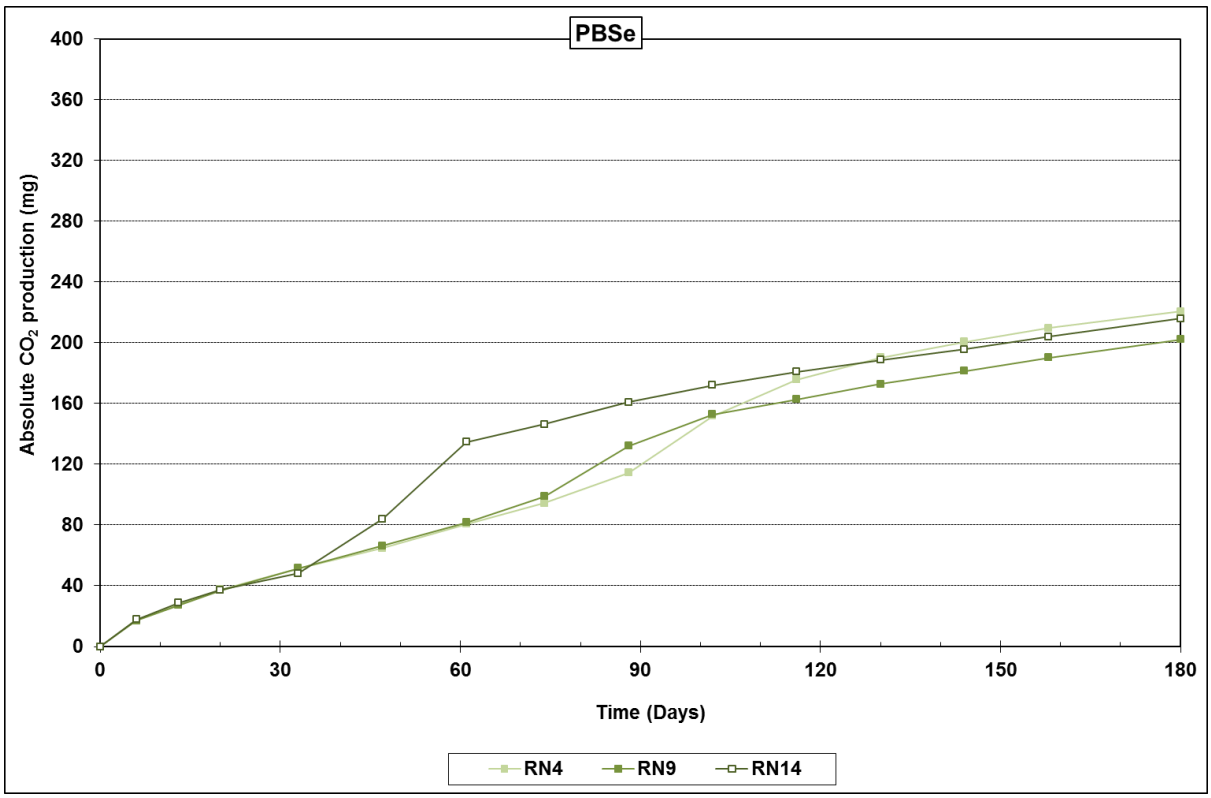


Figure 61. Total CO_2 production in PBSe reactors in seawater (Italy)

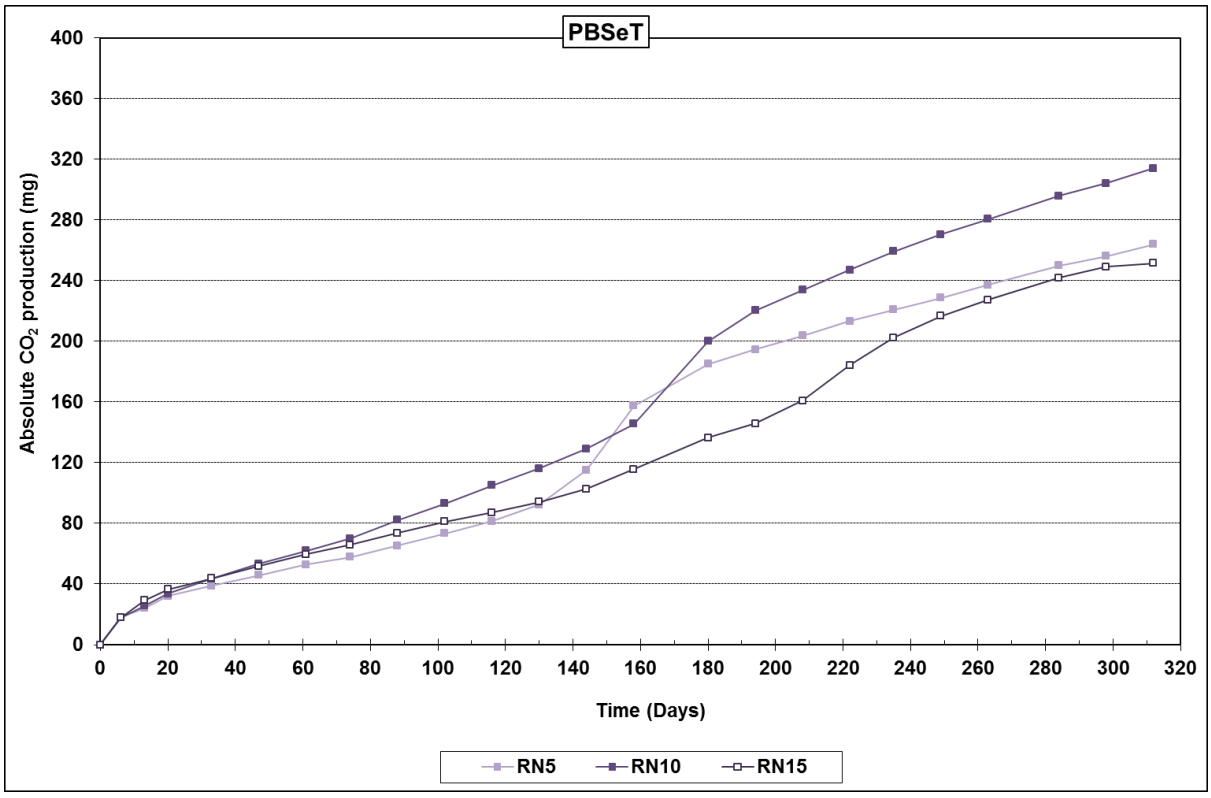


Figure 62. Total CO_2 production in PBSeT reactors in seawater (Italy)

5.3 Laboratory: LeAF

In the first year of test the LeAF laboratory worked with the Italian seawater and sediment from Elba Island, in the second year the tests will be carry out using the seawater and sediment from Greece Salamina Island.

5.3.1 Biodegradation in Eulittoral zone

The set-up of the tests was roughly as prescribed in Annex A. The tests were carried out in 2L Duran® wide mouth bottles equipped with a side port (Figure 5). A container for the CO_2 sorption was connected to the side port. The container was filled with 30 mL of 0.5N KOH solution. The bottle and the container were closed with a „Python“ rubber stopper (Rubber BV, Hilversum, The Netherlands). The bottles were incubated in a closed box that was placed in a room that was kept at 20°C. The test materials (PBSe and PBSeT) were square-shaped specimens with a dimension of approximately 4 cm. The negative control was done with similar 4 by 4 cm specimens of LDPE. For test with PHB as the positive control specimens were cut in 2 by 4 cm pieces (because of the high grammage). The mass of each specimen was recorded.

For the test 15 reactors were prepared to enable testing in triplicate of:

a) Test material 1: PBSe;
b) Test material 2: PBSeT;
c) Reference material – Positive control: PHB;
d) Negative control: LDPE;
e) Blank to correct for endogenous respiration.

Sediment (400 g) was placed at the bottom of each reactor and 30 ml of 0.3N KOH (the CO_2 absorbing solution) was introduced in the container. The reactors were closed and incubated in the temperature controlled room (20 ± 1°C). After 1 week the CO_2 production was measured by titrating the KOH solution with 0.3N HCl. The results showed that the endogenous respiration was similar in the bottles. After the initial 1 week pre-incubation the test was started. For this, the reactors were opened and 100 g of sediment was removed. The surface was smoothened and 2 specimens of test material or LDPE or 1 specimen of PHB was placed on the surface. Thereafter the withdrawn sediment was carefully put on top of the sediment and test material. The specimens were covered with sand in a homogenous layer. Around 100 mg of test material or LDPE or PHB was introduced in the reactors. The blanks for endogenous respiration did not receive any test specimen. The carbon dioxide produced in each reactor reacted with KOH. The amount of carbon dioxide produced is determined by titrating the KOH solution with 0.3 N hydrochloric acid to pH8 and thereafter further to pH 3.8. The amount of CO_2 absorbed was calculated. as described below in section in Annex A. The container for the CO_2 absorber was removed and analysed and titrated before its capacity exceeded. This analysis and replenishment with fresh KOH was

carried out on a regular basis. Each time that the KOH was replaced by a fresh solution, the reactor was weighed to monitor moisture loss from the sediment and allowed to sit open so that the air in the reactor is refreshed before replacing 30 mL of fresh KOH and resealing the reactor. The reactors remained open for approximately 15 min. Distilled or deionized water was added back periodically to the sediment to maintain the initial weight of the reactor. The results of the marine biodegradation tests with sandy marine sediment from Italy are given in Figure 63 (biodegradation) and Figure 64 (CO_2 production). The tests were aborted at 331 days after the start up. The results are in line with the expectations. I.e. the negative control (LDPE) is not degraded, and the net CO_2 production with PHB (positive control) is the highest. Unfortunately one of the PHB triplicates is deviating from the other two replicates. The results of the test materials PBSe and PBSeT (= coded PBSe-co-BT in the figures) are comparable.

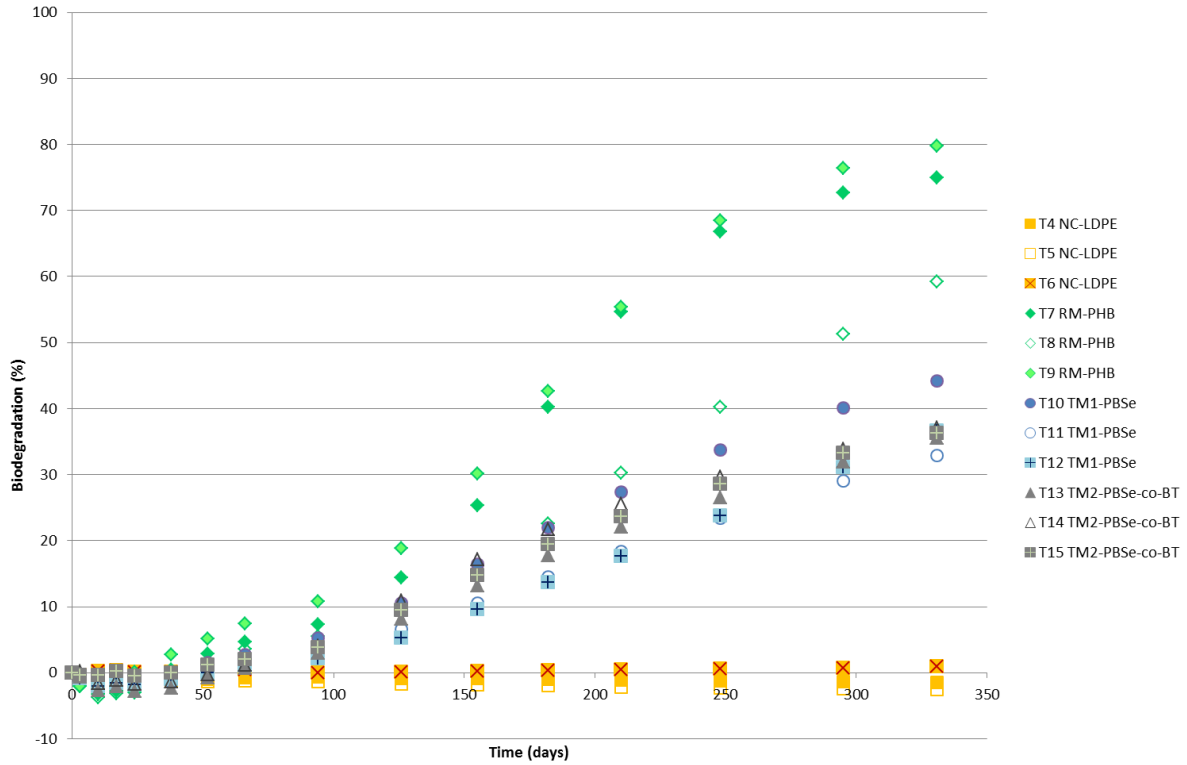
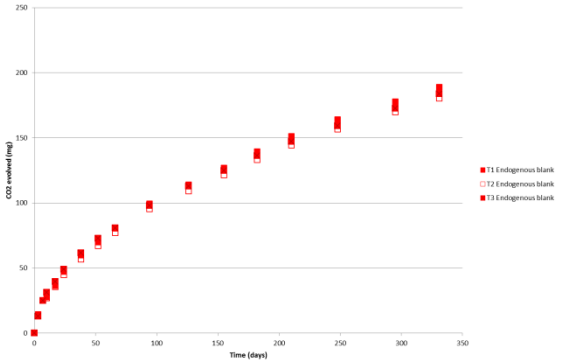
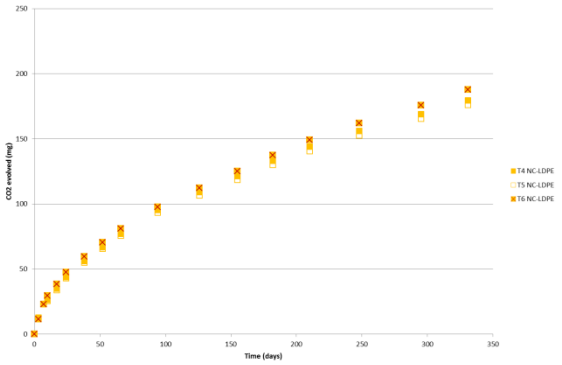


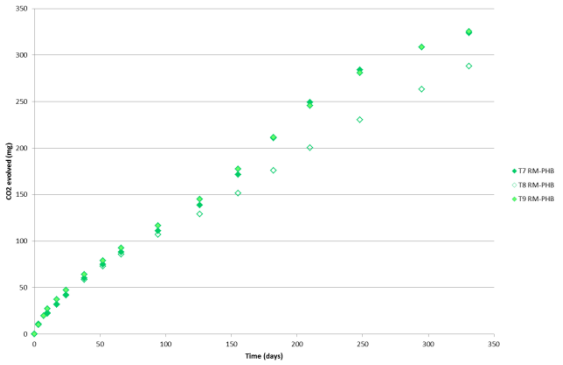
Figure 63. Biodegradation of test materials in sandy marine sediment from Italy.



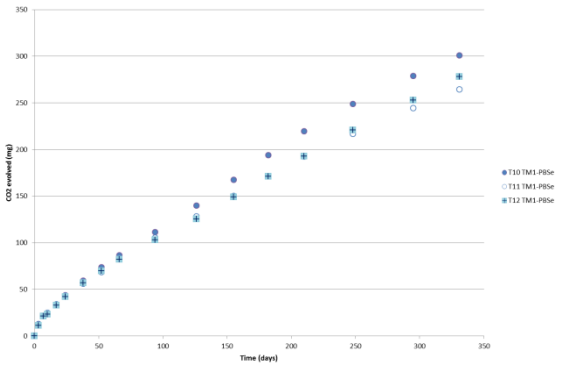
Endogenous blank



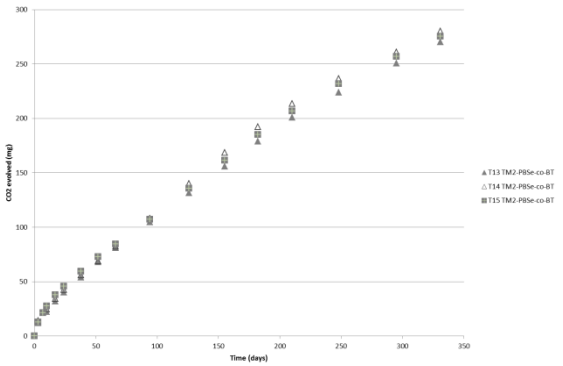
LDPE



PHB



PBSe



PBSe-co-BT

Figure 64. CO_2 production in the reactors with test materials and sandy marine sediment from Italy.

The contents of the bottles were sacrificed at day 331 and the pieces of test item remaining in the test flasks were recovered. The PHB was completely disintegrated and no material could be retrieved from the test flask. For test items PBSe and PBSeT, that was also the case for 2 of the three test flasks. Only in one of each triplicate 25-33 mg (approximately 25-33%) of the initially present material could be retrieved. The pieces of test item retrieved were dried at room temperature but could not always be easily cleaned so in fact some of the weight reported in Table 17 may have been associated with sand or organic material attached to the test items, and in fact the recovery in these cases may even have been lower

(more disintegration). The LDPE was not disintegrated or degraded and completely recovered. Examples of the pieces of test item recovered after the test are given in Figure 65. For LDPE and PHB the % of biodegradation that was measured in the test matches the amount of test item recovered, assuming that around 20% of the PHB converted is retrieved as biomass. For both the PBSe and the PBSeT most of the material had already disintegrated and could not be recovered but only 35-40% of the material originally present was converted to CO_2. Apparently, again assuming 20% assimilation of C in biomass, 40-55% of the material is converted to very small pieces that cannot be observed with the naked eye or retrieved.

Test bottle T4 NC-LDPE (before washing)

Test bottle T13 TM2- PBSeT (after washing)

Figure 65. Test items recovered at the end of the test

Table 17. Results of the pH analysis and recovery of test items at the end of the test at day 331.

Reactor	pH end[a]	Test item start (mg)	Test item end (mg)	Recovery (%)	Biodegradation (%)
T1 Endogenous blank	8.1				
T2 Endogenous blank	8.1				
T3 Endogenous blank	8.2				
T4 NC-LDPE	8.2	107	111	104	-1.0±1.8
T5 NC-LDPE	8.2	102	104	102	
T6 NC-LDPE	8.2	111	104	93	
T7 RM-PHB	8.1	106		0	71.3±10.7
T8 RM-PHB	8.1	100		0	
T9 RM-PHB	8.2	101		0	
T10 TM1-PBSe	8.1	110		0	37.9±5.8
T11 TM1-PBSe	7.9	102	33	33	
T12 TM1-PBSe	8.0	107	3	2	

T13 TM2-PBSeT	8.0	101	25	25	36.3±0.8
T14 TM2-PBSeT	7.9	108		0	
T15 TM2-PBSeT	7.8	105		0	

[a] pH at the start in all bottles was pH 8.0

In principle the test is characterised by an easy test set-up however, there are some issues in the test that could be improved to make the test more realistic. One of the issues are the aerobic conditions that are now imposed by changing the headspace of the test bottles every few weeks. In fact, one may ask whether the conditions are in fact aerobic in the layer that holds the test items (which are buried under 100 grams of sand). Therefore, a thinner sand layer on top of the test items may be more realistic. Also, the amount of test item may be increased to decrease the test duration time and to increase the differences between CO_2 evolved from the endogenous blanks and the amount of CO_2 produced in the active test bottles. As is, the difference may be too small to distinguish between endogenous respiration and actual biodegradation.

5.3.2 Biodegradation in Benthic zone

The set-up of the tests was roughly as prescribed by Annex B. The tests were carried out in 250 ml Erlenmeyer flasks equipped with a container filled with 3 mL 0.5 N KOH for CO_2 sorption (Figure 66). The bottle and the container were closed with a „Python“ rubber stopper (Rubber BV, Hilversum, The Netherlands). The bottles were incubated in a closed box that was placed in a room that was kept at 20°C.

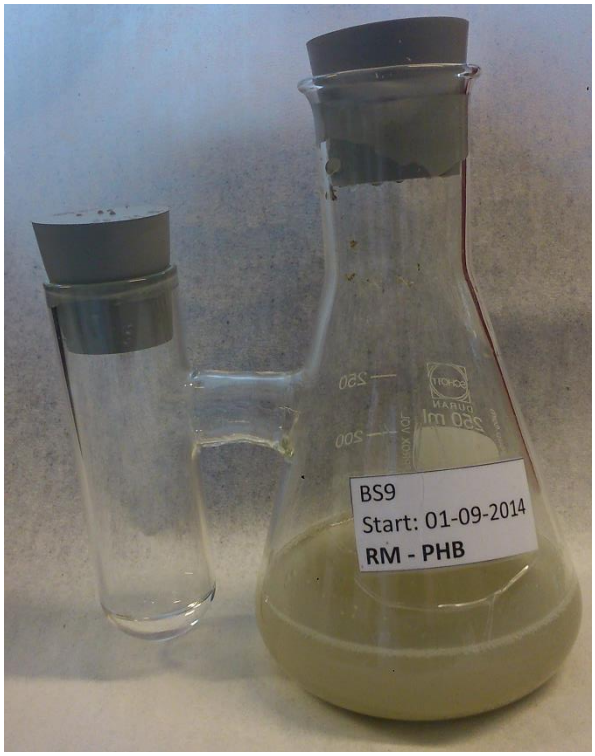


Figure 66. Set up of sandy marine sediment test

The test materials (PBSe and PBSeT) were square-shaped specimens with a dimension of approximately 30 by 30 mm. The negative control was done with a similar specimen of LDPE. For the test with PHB as the positive control specimens were also cut in circles. The mass of each specimen was recorded.

For the test 15 reactors were prepared to enable testing in triplicate of:

a) Test material 1: PBSe;
b) Test material 2: PBSeT;
c) Reference material – Postive control: PHB;
d) Negative control: LDPE;
e) Blank to correct for endogenous respiration.

Sediment (30 g) was placed at the bottom of each reactor with 70 ml seawater and 3 ml of 0.5N KOH (the CO_2 absorbing solution) was introduced in the container. The reactors were closed and incubated in the temperature controlled room (20 ± 1°C). After 1 week the CO_2 production was measured by titrating the KOH solution with 0.1N HCl. The results showed that the endogenous respiration was similar in the bottles. After the initial 1 week pre-incubation the test was started. For this, the reactors were opened and the specimen of test material or LDPE or PHB (in case of negative or positive control, respectively) were placed on top of the sediment. Around 25-35 mg of test material or LDPE or PHB was introduced in the reactors. The blanks for endogenous respiration did not receive any test specimen. Initially no placeholders were used in the test. However, after one week of incubation teflon placeholders were introduced in the bottles (including the endogenous blanks) to keep the test specimens in place. The teflon placeholders were the lower part of an teflon inlay for a GL18 cap (Figure 67). These teflon inlays kept the specimens in place and prevented floating (while they were in place) without applying excess pressure on the samples.

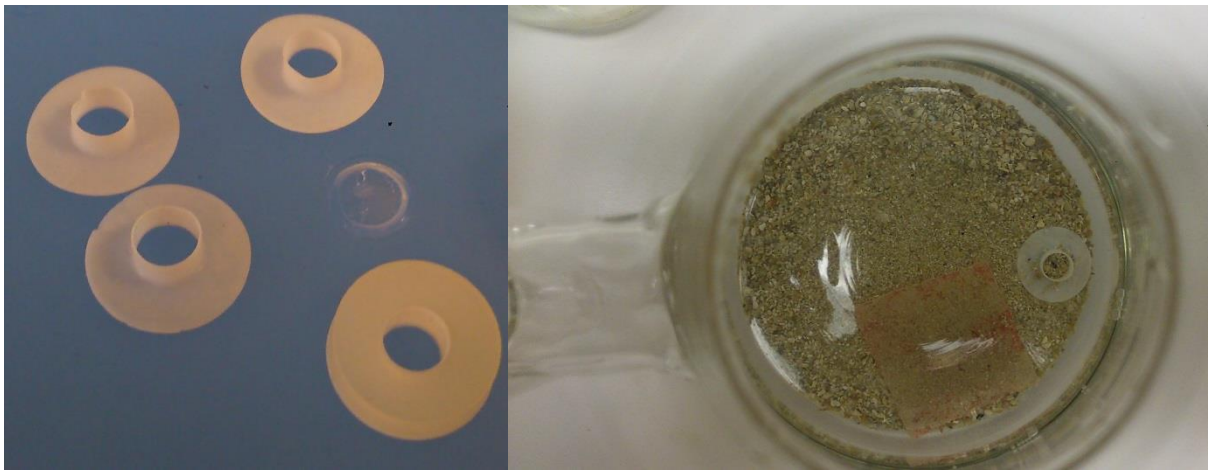


Figure 67. Left photograph: Teflon inlays used for keeping the specimens in place during the first weeks of incubation (left) and original GLXX inlay (of which only lower part was used (right); Right photograph: after prolonged incubation the Teflon inlays were no longer lying on top of specimen, but most specimen were still laying on the sediment.

The carbon dioxide produced in each reactor reacted with KOH. The amount of carbon dioxide produced is determined by titrating the KOH solution with 0.1 N hydrochloric acid to pH 8 and thereafter further to pH 3.8. The container for the CO_2 absorber was removed and analysed and titrated before its capacity exceeded. This analysis and replenishment with fresh KOH was carried out on a regular basis. Each time that the KOH was replaced by a fresh solution, the reactor was weighed to monitor moisture loss from the sediment and allowed to sit open so that the air in the reactor is refreshed before replacing 3 mL of fresh KOH and resealing the reactor. The reactors remained open for approximately 15 min. Distilled or deionized water was added back periodically to the sediment to maintain the initial weight of the reactor. The results of the marine biodegradation tests at the sediment/seawater interface from Italy are given in Figure 68 (biodegradation) and Figure 69 (CO_2 production). The tests have been running for 331 days. Thereafter the tests have been terminated and the contents have been sacrificed for the retrieval of remaining plastic test items. The results are in line with the expectations. I.e. the negative control (LDPE) is not degraded, and the net CO_2 production with PHB (positive control) is the highest. Also, biodegradation appears to have reached a plateau for PHB. There is however a certain uncertainty in the calculation of the biodegradation of PHB due to the possible influence of anaerobic microorganisms on the biodegradation. So far the results of the test materials PBSe and PBST are comparable. Except for the LDPE none of the plastic test items could be retrieved from the test bottles which indicates that disintegration of the test items was complete. In some cases, e.g. for PBSe very small particles were visible but these were the size of sand grains. LDPE was completely recovered from the flasks. PHB was also more or less completely converted to CO_2 as is evidenced by the 81% biodegradation that was calculated from the CO_2 production. The biodegradation percentage of PBSe and PBST was 71% and 76%, respectively, which also indicates that mineralization of these test items was almost complete.

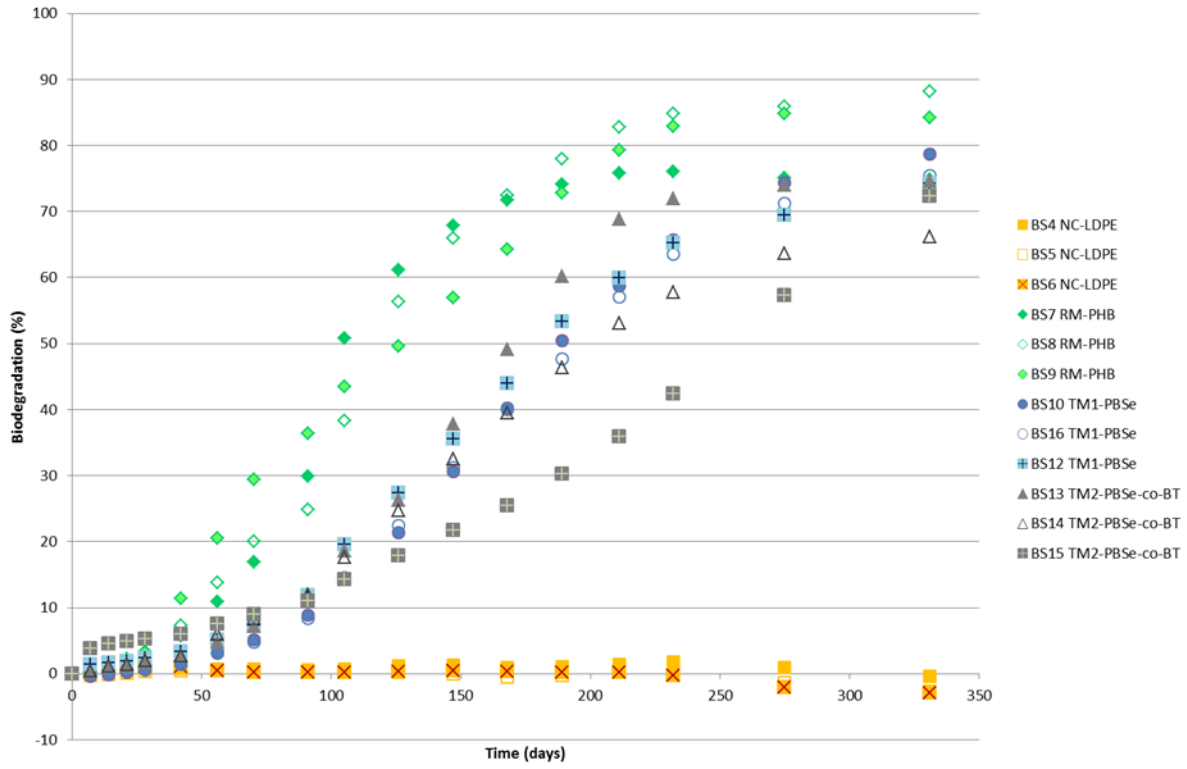
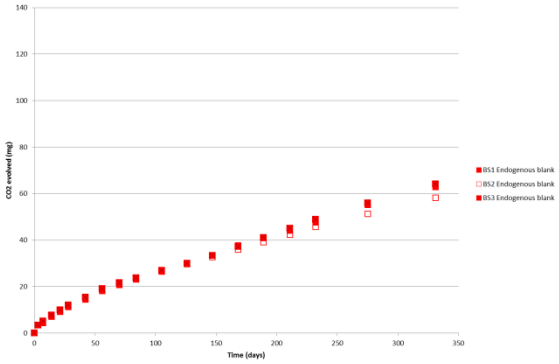
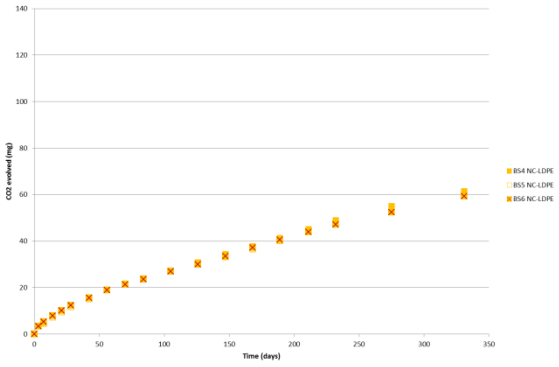


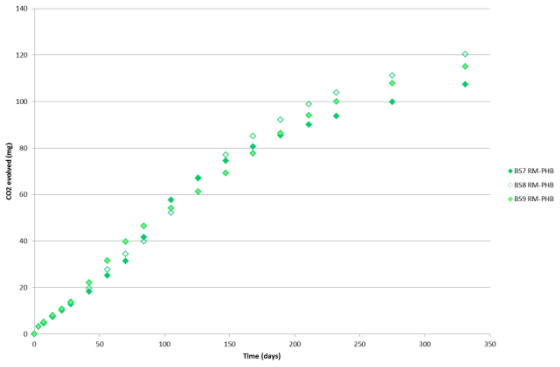
Figure 68. Biodegradation of test materials at sediment/seawater interface from Italy.



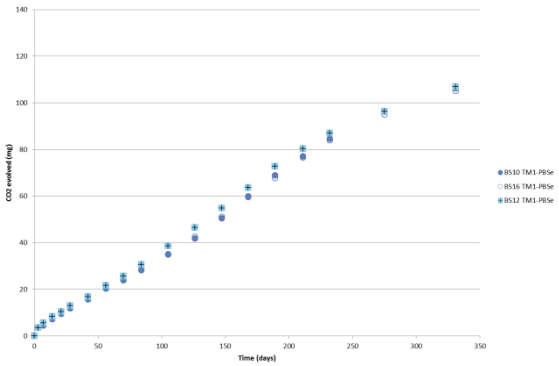
Endogenous blank



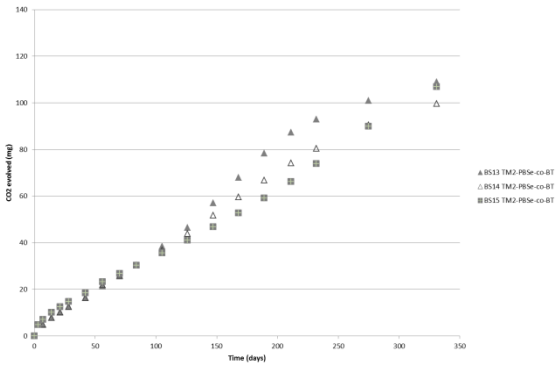
LDPE



PHB



PBSe



PBSe-co-BT

Figure 69. CO_2 production in the reactors with test materials at sediment/seawater interface from Italy.

Table 18. Results of the pH analysis and recovery of test items at the end of the test at day 331.

Bottle	pH start	pH end[a]	Test item start (mg)	Test item end (mg)	Recovery (%)	Biodegradation (%)
BS1 Endogenous blank	8.0	8.4				
BS2 Endogenous blank	8.1	8.5				
BS3 Endogenous blank	8.0	8.6				
BS4 NC-LDPE	8.1	8.5	27.29	28.00	103	-2.0±1.4
BS5 NC-LDPE	8.1	8.4	24.43	26.20	107	
BS6 NC-LDPE	8.0	8.4	25.86	28.20	109	
BS7 RM-PHB	8.1	8.5	34.76	0	0	82.4±6.9
BS8 RM-PHB	8.0	8.5	37.92	0	0	
BS9 RM-PHB	8.0	8.5	36.15	0	0	
BS10 TM1-PBSe	8.0	8.4	23.63	0	0	76.1±2.3
BS16 TM1-PBSe	8.0	8.4	24.06	0	0	
BS12 TM1-PBSe	8.1	8.4	25.41	0	0	
BS13 TM2-PBSeT	8.0	8.4	26.46	0	0	71.0±4.3
BS14 TM2-PBSeT	8.1	8.4	23.94	0	0	
BS15 TM2-PBSeT	8.0	8.4	26.13	3.50	13	

Suggestions for improvements to the set-up of the test:

- Nature of cover slip; a cover slip, which is too light will have little if any effect, but with a heavy coverslip there will an influence of applied pressure that is difficult to quantify. The cover slip that was now used by LeAF initially kept the samples in place, but moved when the test flasks were slightly moved which made it difficult to keep the samples in place. However, after prolonged incubation biofilms started to develop on the test items and the items remained laying on the sediment without further external influence.
- Anaerobic conditions were prevailing underneath the sample and at the bottom of test bottles during a certain period of the test period as evidenced by the appearance of black spots in the test flasks directly underneath the PHB test items (Figure 70). This black stain formation is probably related to the formation of sulphide precipitates. This suggests that there is a lack of oxygen around the more rapidly degraded test items. It is therefore uncertain whether the % of biodegradation is accurately calculated in the case of PHB since the biodegradation may also at least partially be carried out by anaerobic (sulphate reducing) bacteria. This staining did not occur with LDPE or test items 1 and 2. Mild shaking to induce oxygenation of the top layer of the sediment may be beneficial to sustain aerobic conditions throughout the test period.

Figure 70. Formation of black spots in the sediment

- Amount of sample: the amount of test item may be increased to decrease test duration time and to increase the differences between CO_2 evolved from the endogenous blanks and the amount of CO_2 produced in the active test bottles.
- Addition of nutrients to decrease test duration: the addition of nutrients may lead to faster transformation of the test items.

5.4 Laboratory: AUA

5.4.1 Biodegradation in Eulittoral zone

Experimental procedure

Sandy sediment was collected from the eulittoral zone of the shoreline in Greece (Salamis area), where the sediment is submerged in seawater at times due to waves.The sediment was filtered so as to remove coarse organic or inorganic articles in order to obtain a homogeneous sandy substrate. The properties of the sediment used for the eulittoral lab tests were determined at the lab of Soil Science and Agricultural Chemistry of AUA and are presented in Table 3.

Approximately 356 g of sediment is placed in the bottom of each vessel. Glass vessels of approximately 4L volume that can be sealed air-tight are used. The sediment is enriched with nitrogen (by adding the appropriate weight of fertilizer to a C:N ratio equal to 10:1 (w/w)). The same amount of nutrients was also added in blanks. The weight of all vessels is recorded and the samples of the test materials are added (approximately 1000 mg organic carbon). The samples are placed in the vessels in the following way: about 100 g of sediment is removed from the layer in the bottom of the reactor. This sediment is kept in a clean container. The test specimen is laid down on top of the remaining sediment. No specimen is placed in the blank reactors. The withdrawn sediment ıs put back in the reactor to form a homogenous layer that covers the specimens. For this experiment 3 blanks, 3 PHB samples, 3 PBSe and 3 PBSeT samples were tested. The initial weight of the reactors ıs recorded. The CO_2 absorbing solution and water are introduced in beakers containing 50 ml KOH 1N and 50 ml distilled water and the vessels are sealed and placed in a darkened chamber or cabinet, where the temperature is maintained between 25 ± 2 °C. The

experiment did not include a preliminary phase. The amount of CO_2 produced is determined by titrating the remaining potassium hydroxide with 0.25 N hydrochloric acid. The container for the CO_2 absorber is removed and titrated twice per week for the first 2 to 3 weeks and every 1 to 2 weeks thereafter. When the CO_2 absorbers are removed, the reactors are allowed to stay open so that the air is refreshed before replacing 50 ml of fresh potassium hydroxide and resealing the reactor. The reactors remain open for approximately 10 min. The sediment used for the tidal lab tests was collected from the Salamis area and the properties of the sediment were determined. The parameters of the eulittoral lab test are summarized in Table 5.

5.4.1.1 Results

Examination of the addition of fertilizer effect

Firstly, the effect of the addition of nutrients in the system was examined. Two PHB samples were used (1000 mg organic carbon per sample) in two different reactors. Fertilizer was added in one of the reactors by enriching the sandy sediment with the appropriate amount of N corresponding to N:C ratio 1:10 of the sample's organic carbon. The results (Figure 71) showed that the addition of fertilizer strongly affects the biodegradation rate. Thus for 50 days the system without fertilizer reached a level of 6.4% biodegradation while the system with the fertilizer reached 50.6% biodegradation. After 106 days the biodegradation degree was 14% for the PHB without fertilizer while it was 72.5% for the PHB with fertilizer. Finally after 127 days the biodegradation was 18.4% and 75.2% for the PHB without fertilizer and for the PHB with fertilizer, respectively. Thus it was decided that the addition of fertilizer is an important factor that must be consider for the designing of the experiment.

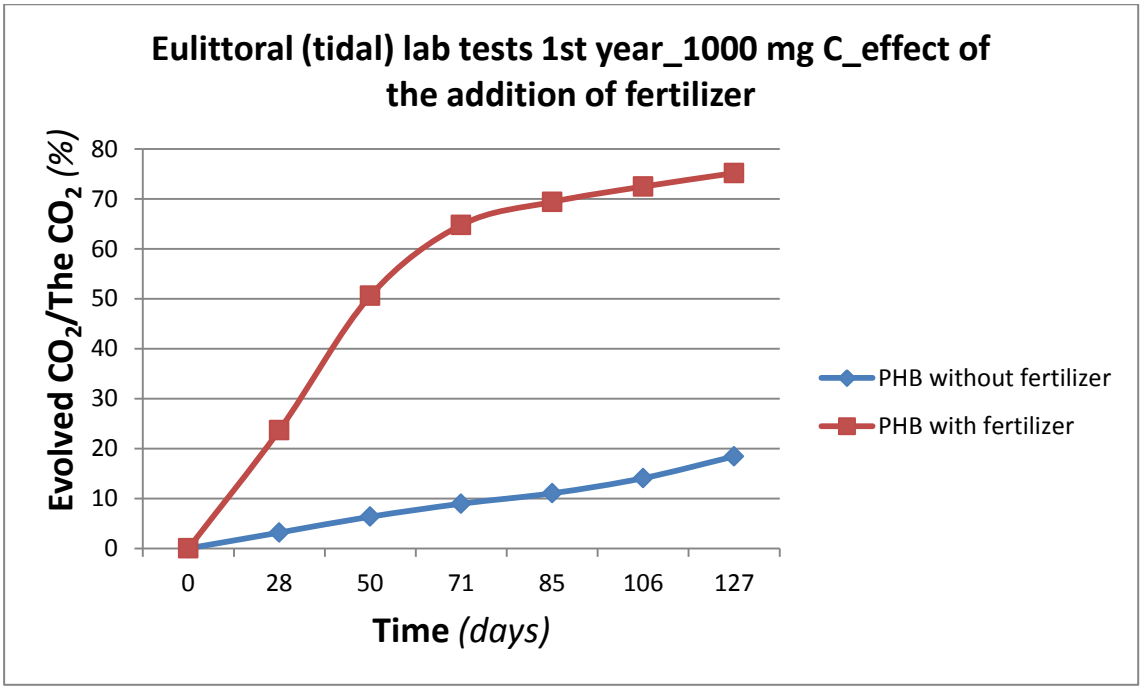


Figure 71. Effect of the addition of nutrients in the eulittoral test

PHB, PBSe, PBSeT (1000 mg organic carbon)

Following the above described preliminary tests, the three studied materials, PHB, PBSe, and PBSeT were tested. The exact weights and corresponding organic carbon content of the samples are presented in Table 19. The results are summarized in Figure 72 and Figure 73.

Table 19. Weights and corresponding organic carbon of materials used in the eulittoral test

Material	Weight of sample (g)	Organic carbon in sample (g)
PHB 1	2.0949	1.0018
PHB 2	2.0871	0.9980
PHB 3	2.1098	1.0090
PBSe 1	1.5370	1.0030
PBSe 2	1.5162	0.9895
PBSe 3	1.5116	0.9865
PBSeT 1	1.5190	0.9911
PBSeT 2	1.5550	1.0146
PBSeT 3	1.5540	1.0140

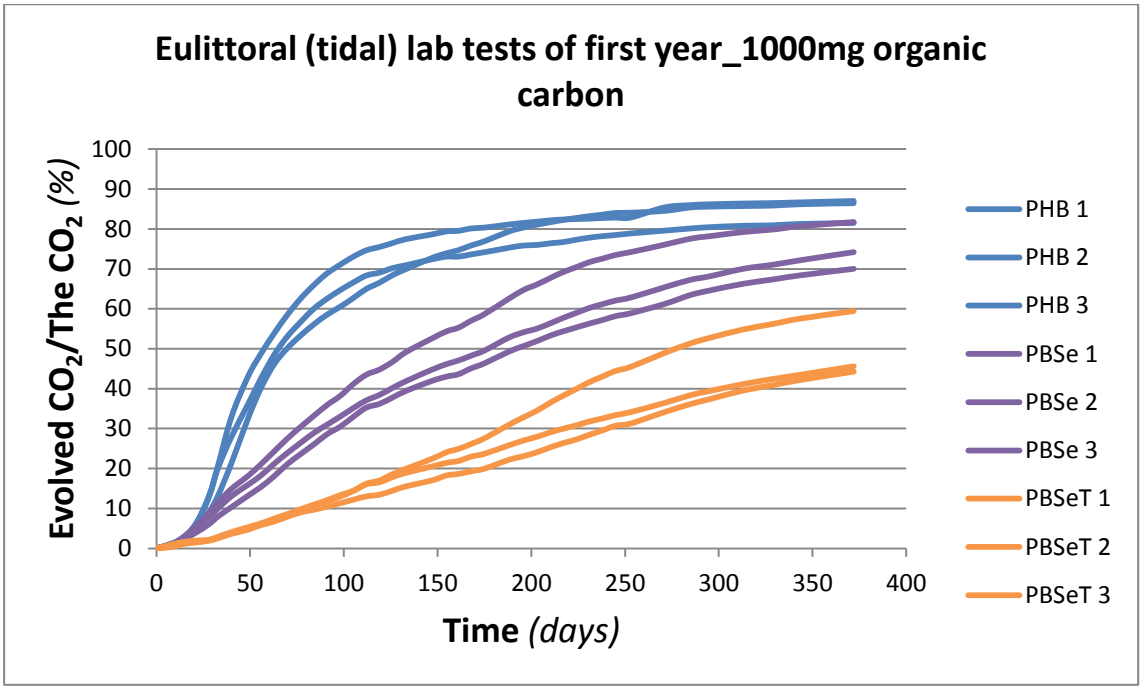


Figure 72. Biodegradation vs. time for eulittoral test (1000 mg carbon)

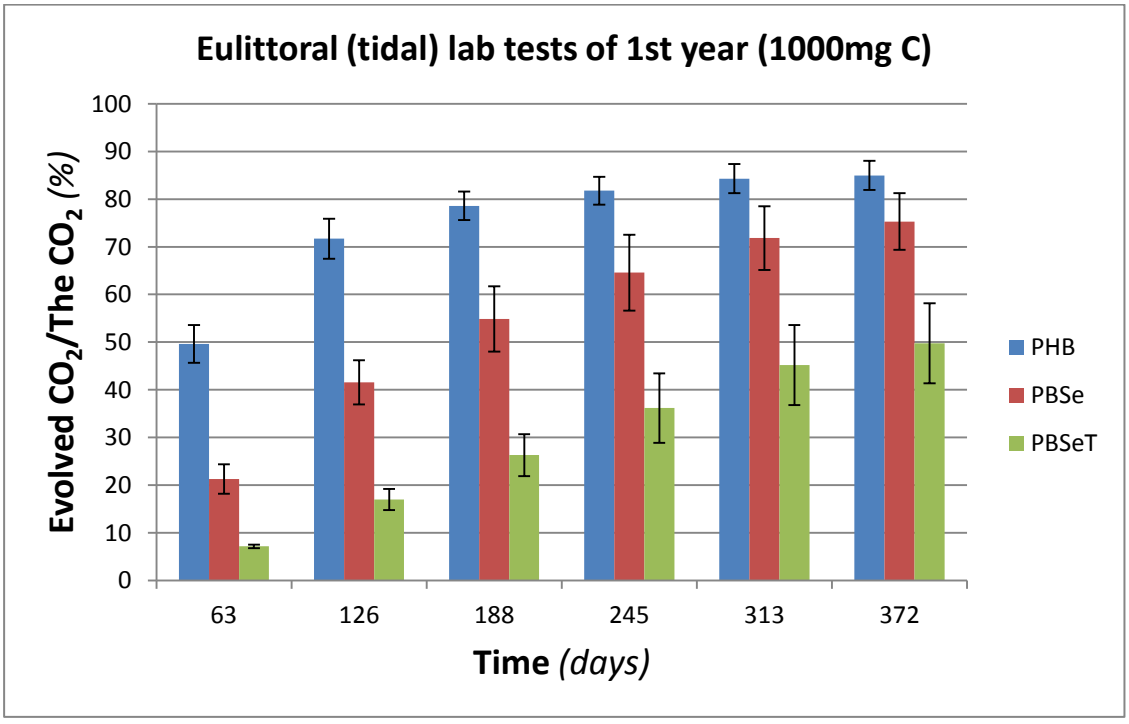


Figure 73. Biodegradation vs. time for eulittoral test (1000 mg carbon)

The biodegradation average values (%) for 63, 126, 188, 245, 313 and 372 days are summarized in Table 20.

Table 20. Average values and standard deviation of % biodegradation of the materials vs time

Days	PHB	PBSe	PBSeT
63	49.6 ± 4.0	21.3 ± 3.1	7.2 ± 0.4
126	71.7 ± 4.2	41.6 ± 4.6	17.0 ± 2.2
188	78.6 ± 3.0	54.9 ± 6.9	26.3 ± 4.4
245	81.8 ± 2.9	64.6 ± 8.0	36.2 ± 7.3
313	84.3 ± 3.1	71.8 ± 6.7	45.2 ± 8.4
372	85.0 ± 3.0	75.3 ± 5.9	49.8 ± 8.4

The results showed that after 372 days of monitoring the biodegradation for the PHB reached a value of 85% ± 3.0%, for the PBSe 75.3% ± 6.0% and for the PBSeT 49.8% ± 8.4%. The results showed good reproducibility with low values of standard deviation thus it was decided that the same experimental protocol should be used for next series of the eulittoral lab test experiments.

5.4.2 Biodegradation in Benthic zone

Experimental procedure

Seawater and sediment from the sea bottom had been sampled separately near the shoreline in Salamis island area in Greece in 18-20 m depth. The properties of the sediment used for the sublittoral lab tests were determined at the Soil Science and Agricultural Chemistry laboratory of AUA and are presented in Table 3. The sediment and seawater were transported and stored at approximately 4°C. The experimental procedure for the sublittoral tests is as following: 170 g of wet sediment are placed on the bottom of each reactor. Then 380 ml of natural seawater is added. The carbon dioxide absorber consists of 50 ml of KOH 1N. The flasks are kept at constant temperature 25°C and the CO_2 evolution is monitored. The plastic film samples are placed on the sediment-water interphases of the reactors and are covered by a thin layer of sediment in order to keep them underwater. The sample quantity depends on the organic content of the material so as to correspond to the same selected amount of organic carbon. The mass of the sediment, the mass of the specimen and the volume of seawater added, for each vessel is recorded. This experiment did not include a preliminary phase. The amount of CO_2 produced is determined by titrating the remaining potassium hydroxide with 0.25 N hydrochloric acid by automatic titrator. The CO_2 absorbers are titrated every 4 days for the first 2 to 3 weeks and every 1 to 2 weeks thereafter. At the time of removal of the containers, the reactor is allowed to stay open so that the air is refreshed before replacing 50 ml of fresh potassium hydroxide and resealing the reactor. The reactors remain open approximately for 15 min. Usually, when a constant level of CO_2 evolution is attained (plateau phase reached) and no further biodegradation is expected, the test is considered to be completed.

The parameters of the sublittoral lab test are summarized in Table 6.

Effect of the addition of fertilizer in the system

The effect of the fertilizer addition to the system was examined by running a special experiment with monitoring the biodegradation of PHB sample of 80 mg organic carbon without fertilizer and the biodegradation of a PHB sample in a similar reactor but with the addition of fertilizer. The experiment showed (Figure 74) that the addition of fertilizer strongly affects the biodegradation degree by enhancing the biodegradation rate (% biodegradation reached approximately 80% for the PHB with the fertilizer while for the PHB without fertilizer was approximately 60% after a period of 76 days).

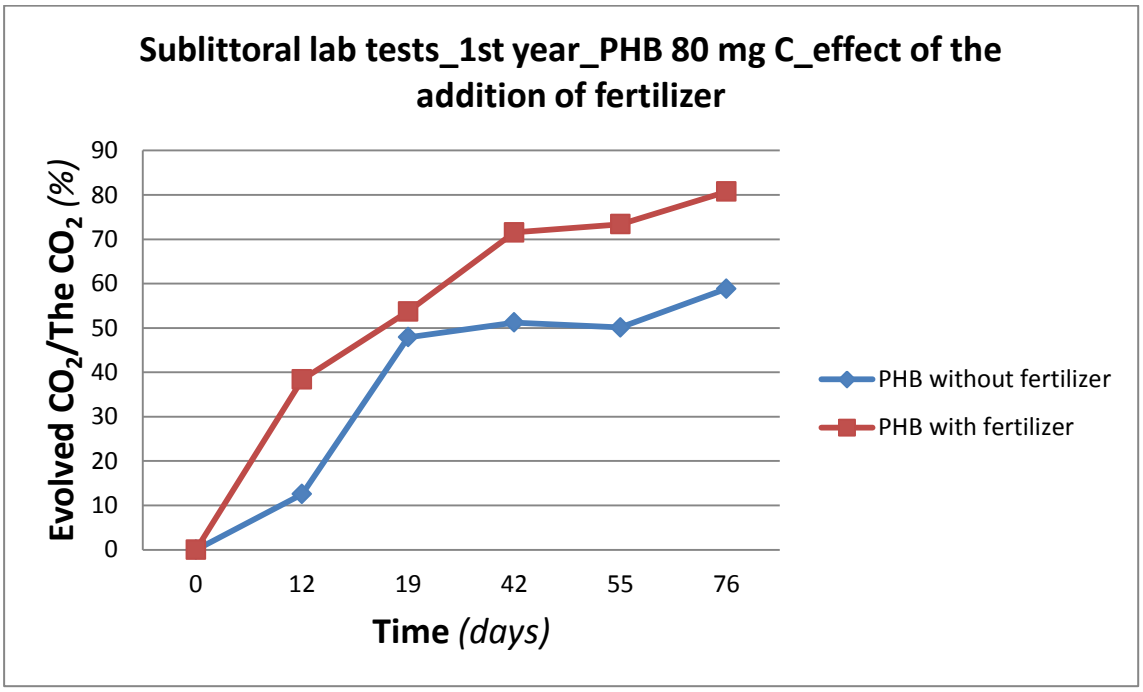


Figure 74. Effect of addition of nutrients in the sublittoral lab test

Experiment 1. PHB, PBSe and PBSeT (80 mg organic carbon)

The first series of experiments for the sublittoral environment were performed by using 80 mg of organic material for each material. That means approximately 165 mg sample for the PHB material (47.82% organic carbon content) and approximately 125 mg sample for the PBSe and PBSeT materials (organic carbon content 65.26% and 65.25% respectively) (Table 21). The experiment included 3 blanks, 3 PHB, 3 PBSe and 3 PBSeT replicates. The fertilizer used to enrich the sediment in nutrients had composition N-P-K: 13-2-44. It includes nitrogen in nitric form. The weight of the fertilizer used was calculated based on a ratio of N/C equal to 1/10. The appropriate amount of fertilizer was dissolved in natural seawater and added to each reactor. As mentioned above 170 g of sediment was added to each reactor and the volume of natural seawater plus the natural seawater with the fertilizer added in each reactor was approximately 400 ml. The CO_2 production determined by titration was

monitored for a period of 286 days. The analytical results are presented in Figure 75 and Figure 76.

Table 21. Weights and corresponding organic carbon of materials

Material	Weight of sample (g)	Organic carbon in sample (g)
PHB 1	0.1697	0.080
PHB 2	0.1641	0.078
PHB 3	0.1661	0.079
PBSe 1	0.1275	0.083
PBSe 2	0.1262	0.082
PBSe 3	0.1252	0.081
PBSeT 1	0.1262	0.082
PBSeT 2	0.1230	0.080
PBSeT 3	0.1265	0.082

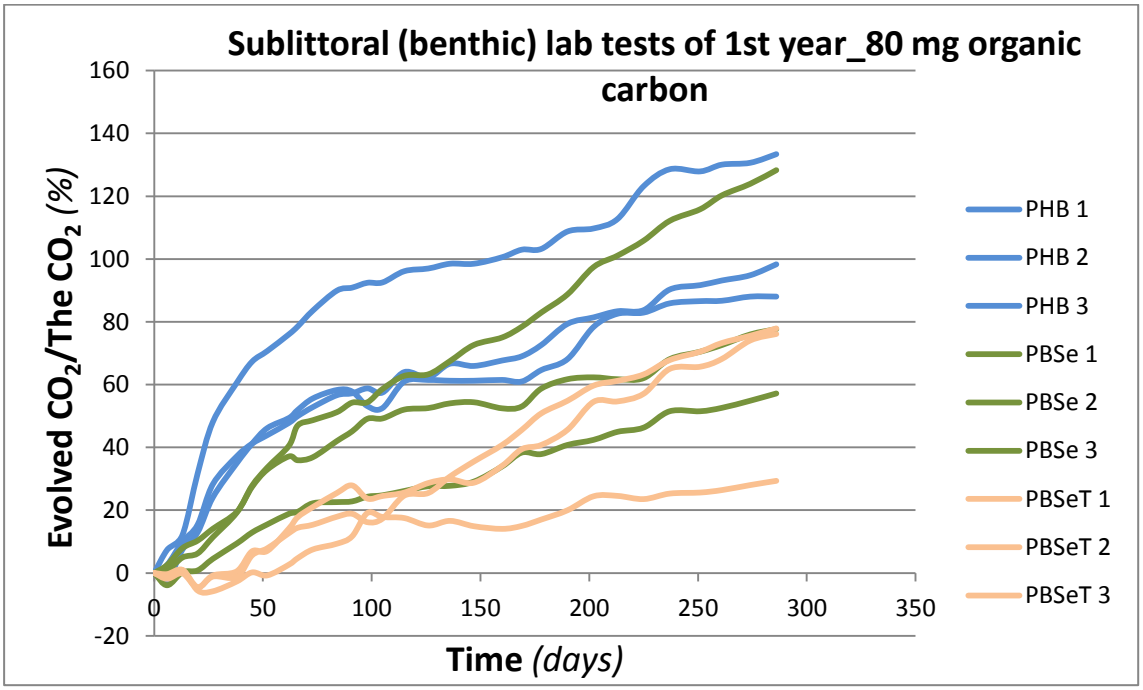


Figure 75. Biodegradation (%) vs time for the 1st sublittoral test (80 mg carbon)

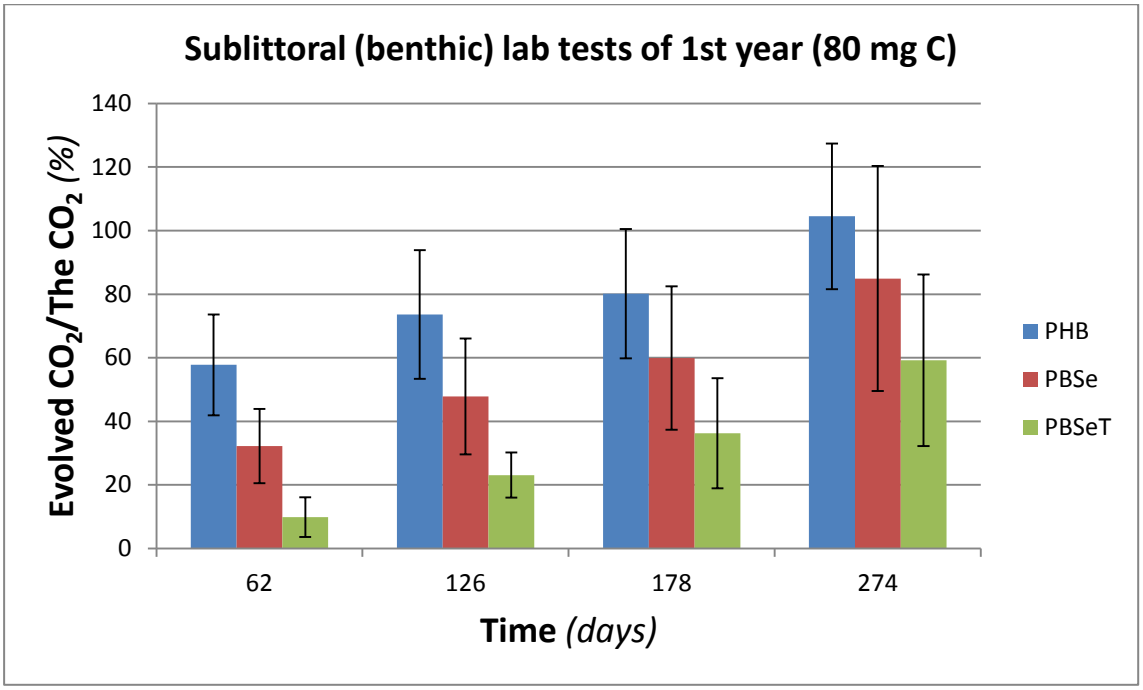


Figure 76. Biodegradation (%) vs time for the 1st sublittoral test (80 mg carbon)

After 274 days a high degree of evolved CO_2/theoretical CO_2 was reached for the PHB samples exceeding 100% (104.5% average ±22.9% (sdev)), for the PBSe samples 84.9% ± 35.4% and for the PBSeT samples 59.2% ± 27.0%. The standard deviation values were high and the results were considered unreliable. The high variability of the results was attributed to the low amount of organic carbon used for the experiment. The following step was to use a higher amount of samples organic carbon and evaluate the new results.

Experiment 2. PHB, PBSe, PBSeT (500 mg organic carbon)

The next series of experiments for the benthic environment were performed by using 500 mg of organic carbon for each material. That means approximately 1050 mg sample for the PHB material and approximately 750-770 mg sample for the PBSe and PBSeT materials (Table 22). The experiment did not includes replicates. The fertilizer used for the amendment of sediment, was of the same N-P-K: 13-2-44 type than used in the previous experiment. The weight of fertilizer used was based on the ratio of N/C equal to 1/10. The appropriate amount of fertilizer was dissolved in natural seawater and added to each reactor. As mentioned above 170 g of sediment was added to each reactor and the volume of natural seawater plus the natural seawater with the fertilizer added in each reactor was approximately 400 ml. The CO_2 production determined by titration was monitored for a period of 286 days. The analytical results are presented in Figure 77.

Table 22. Weights and corresponding organic carbon of materials

Material	Weight of sample (g)	Organic carbon in sample (g)
PHB	1.0489	0.5016
PBSe	0.7702	0.5026
PBSeT	0.7502	0.4895

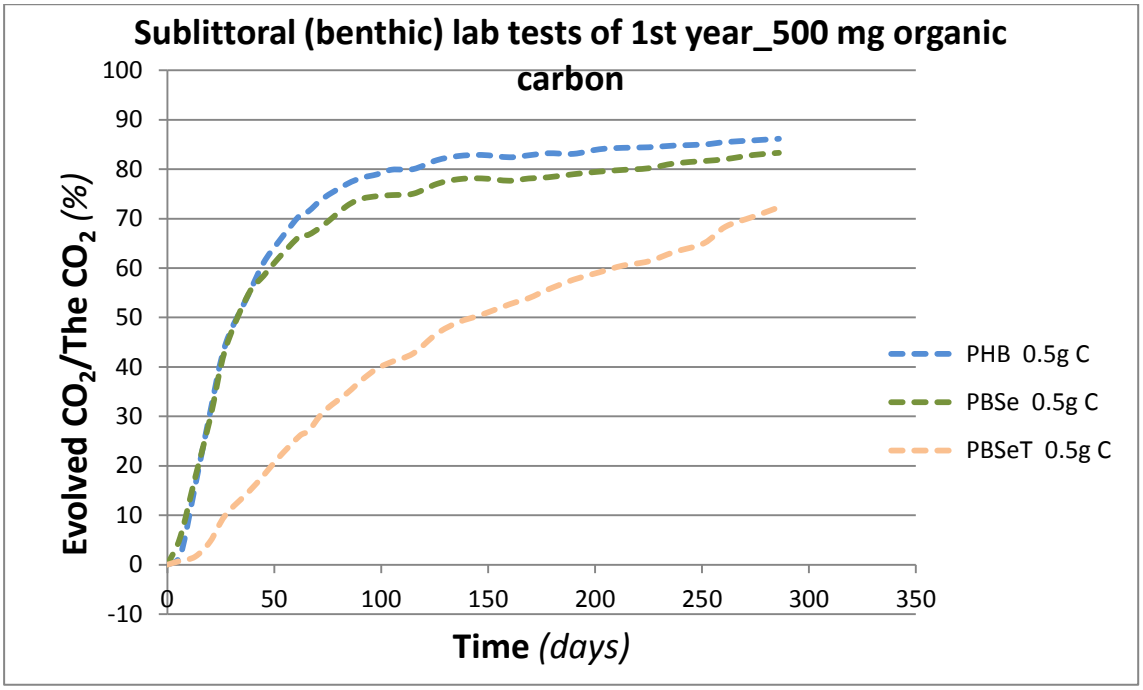


Figure 77. Biodegradation (%) vs time for the 2nd sublittoral test (500 mg carbon)

The results showed that after 274 days the degree of evolved CO_2/ theoretical CO_2 for the PHB reached a value of 85,8%, for the PBSe 82,9% and for the PBSeT 70,4%. These results were considered as more reliable than when using 80 mg of organic carbon though only one replicate was performed for each test material. It was decided to perform another experiment using intermediate quantities of organic carbon in order to determine the optimum quantity of organic carbon for the benthic test.

Experiment 3. PHB (200, 300 and 500 mg organic carbon)

This experimental trial included only PHB material in different weights. The inoculum (sediment), the seawater as well as the nutrients used followed the procedure used for the first two trials. Three different sample quantities were used corresponding to a) 200 mg organic carbon (2 replicates), b) 300 mg organic carbon (2 replicates) and c) 500 mg organic carbon (2 replicates) (Table 23). Two blanks were also used. The biodegradation was monitored for 50 days and for this time the highest degree of biodegradation was reached for the PHB materials containing 300 mg of organic carbon (approximately 55% for PHB containing 300 mg organic carbon, 33% for PHB containing 200 mg organic carbon and 44%

for PHB containing 500 mg organic carbon). The results are presented in Figure 78. Thus it was decided that the optimum quantity of material to be used is approximately 300 mg organic carbon per sample.

Table 23. Weights and corresponding organic carbon of materials

Material	Weight of sample (g)	Organic carbon in sample (g)
PHB 1	0.4170	0.1994
PHB 2	0.4179	0.1998
PHB 3	0.6242	0.2985
PHB 4	0.6275	0.3001
PHB 5	1.0467	0.5005
PHB 6	1.0466	0.5005

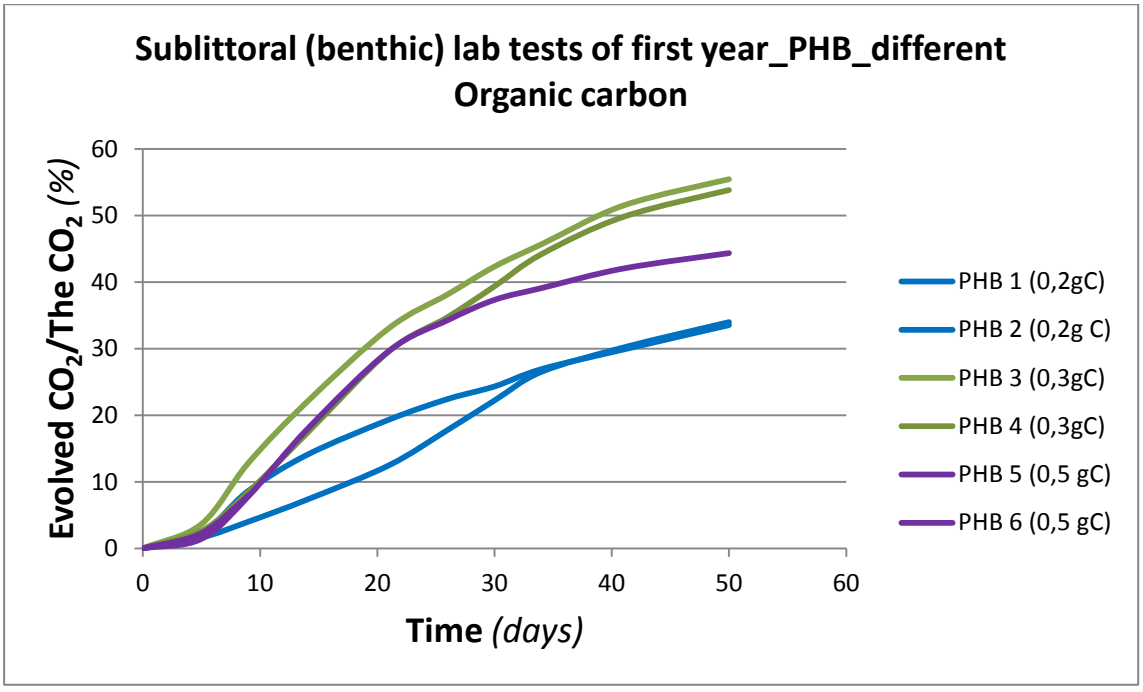


Figure 78. Biodegradation (%) vs time for the 3rd sublittoral test (200, 300 and 500 mg carbon)

5.4.3 Biodegradation in pelagic zone

Oxytop® system Experimental procedure

A sample of seawater was collected near Salamis Island, Greece. The seawater was stored at approximately 4°C. There was no need to remove plant pieces, sea shell or other large articles because of the purity of the seawater. The reactors used were glass flasks of 250 ml. The experimental procedure can be described as follows: 100 ml of natural seawater are placed into each 250 ml bottle. The test material (in the form of cut pieces) is put in the

seawater. The water in all flasks is stirred by magnetic stirrers and it is incubated at a temperature of 25 °C. Fertilizer of the type 'F2'(nutrients in the form of $NaNO_3$ is added to the seawater in each flask based on the ratio of N/C: 1/10. A plastic container with 6 ml KOH 1N is added for the trapping of CO_2. The plastic container with the KOH is placed in the reactor above the seawater. The flask lid after the placement of the corresponding specimen and trap is screwed on and the experiment starts after attaching all of the respirometry bottles to the pressure measuring devices. The flasks are aerated frequently. Oxygen consumption is measured by the Oxytop® manometric system. Titrations are also performed during the test in order to compare the biodegradation rates obtained by oxygen consumption and CO_2 production measurements. Once the system has started the O_2 consumption is continuously monitored by recording the pressure in the flasks every two hours. The duration of the experiments is 90 days. The tests did not include a preliminary phase. Titrations are performed every 5 days for the first 2-3 weeks and once per week thereafter. Before placing a new solution of KOH, the reactors remained open for about 15 minutes allowing the air in the reactors to refresh. The CO_2 traps are titrated with an HCl solution of 0.25 N. The parameters of the pelagic lab test are summarized in Table 7.

PHB, PBSe, PBSeT, 100 mg sample

The first experiment performed for the 1st year of pelagic lab tests examined the biodegradation in the pelagic environment of the three materials (PHB, PBSe, PBSeT) using 100 mg of sample for each material. The replicates included 3 blanks, 3 PHB samples, 3 PBSe samples and 3 PBSeT samples. The exact weights of samples and the corresponding organic carbon content of samples are presented in the Table 24. For the experiment the biodegradation values based on continuous pressures measurements and the biodegradation values based on titrations were determined. The BOD results and the titrations results presented discrepancies. The BOD results are considered unreliable because of high standard deviation values and they are presented in Figure 79. The titration results are presented in Figure 80. The biodegradation rates after 27 days of are summarized in Table 25.

Table 24. Weights and corresponding organic carbon of the first pelagic lab test experiment

Material	Weight of sample (mg)	Organic carbon in sample (g)
PHB 1	106.9	0.0511
PHB 2	108.8	0.0520
PHB 3	109.0	0.0521
PBSe 1	104.9	0.0684
PBSe 2	106.8	0.0697
PBSe 3	108.1	0.0705
PBSeT 1	109.0	0.0711
PBSeT 2	108.6	0.0709
PBSeT 3	105.9	0.0691

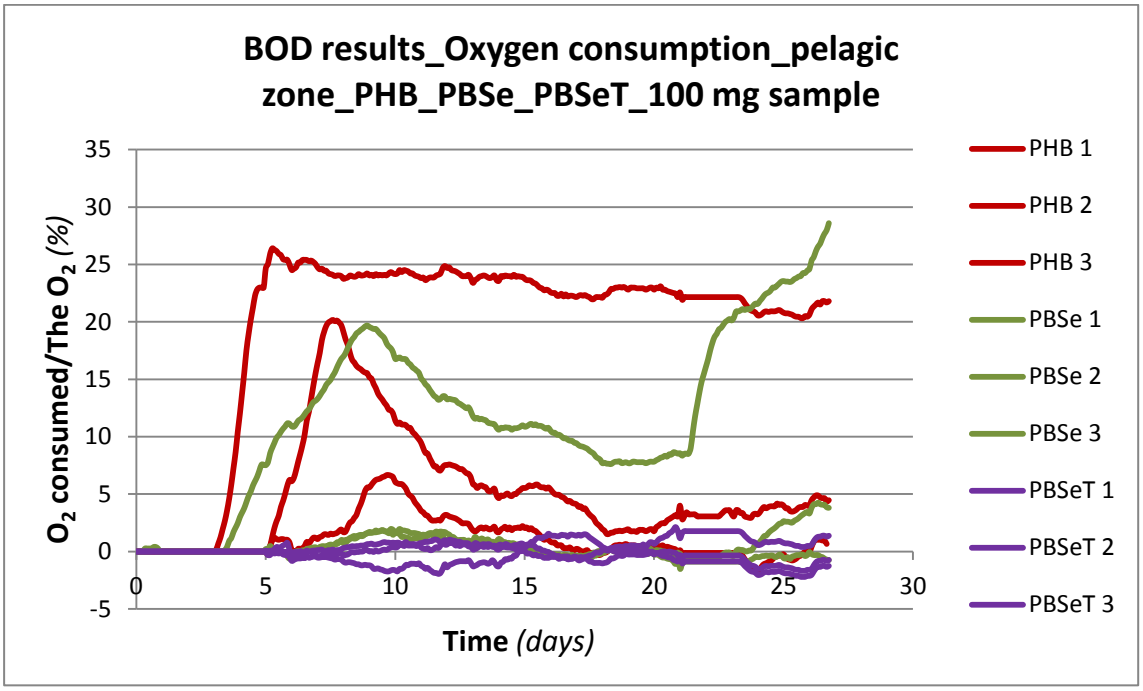


Figure 79. BOD results (O_2 consumption) biodegradation (%) vs. time for the pelagic lab test (100 mg sample)

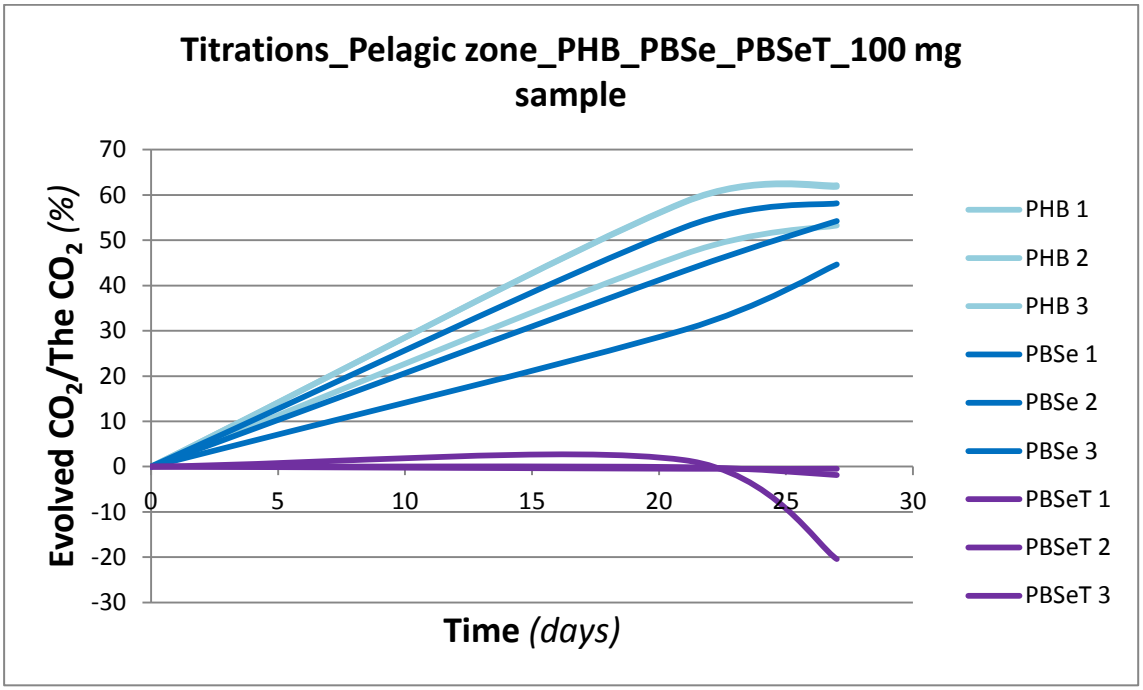


Figure 80. Titrations results biodegradation (%) vs. time for the pelagic lab test (100 mg of sample)

Table 25. Average biodegradation (%) and standard deviations of materials after 27 days

	PHB	PBSe	PBSeT
Average biod (%)	59.1±5.0	52.3±6.9	-7.6±11.1

The biodegradation follows the order PHB>PBSe>PBSeT. Since the BOD results were not reliable and for the titrations the standard deviations were also relatively high it was decided to repeat the experiment by using a larger sample quantity.

PHB, PBSe, PBSeT, 300 mg sample

The following experiment performed for the 1st year of pelagic lab tests examined the biodegradation in the pelagic environment of the three materials (PHB, PBSe, PBSeT) using 300 mg of sample for each material. The replicates included 3 blanks, 3 PHB samples, 3 PBSe samples and 3 PBSeT samples. The exact weights of samples and the corresponding organic carbon content of samples are presented in Table 26. For the experiment the biodegradation rates based on continuous pressure measuring and those based on titrations were determined. Similarly to the previous experiment the BOD results were considered unreliable (high standard deviation values) and are presented in Figure 81. The results of CO_2 evolution are presented in Figure 82 and Figure 83. The results from the two methods (BOD versus CO_2 evolution) presented a discrepancy. The biodegradation rates from oxygen consumption based on pressure change are significantly lower than the biodegradation values determined by titration method (Figure 81 versus Figure 82). The CO_2 evolution results appear to be reliable in terms of the low standard deviations but the higher rates of biodegradation reached a plateau at approximately 70% biodegradation (PHB and PBSe samples). The PBSeT samples showed almost zero values of biodegradation for the whole period of the experiment.

Table 26. Weights and corresponding organic carbon of materials

Material	Weight of sample (mg)	Organic carbon in sample (g)
PHB 1	304.6	0.1457
PHB 2	300.6	0.1438
PHB 3	302.0	0.1444
PBSe 1	306.5	0.2000
PBSe 2	306.5	0.2000
PBSe 3	304.4	0.1987
PBSeT 1	302.3	0.1972
PBSeT 2	301.6	0.1968
PBSeT 3	303.6	0.1981

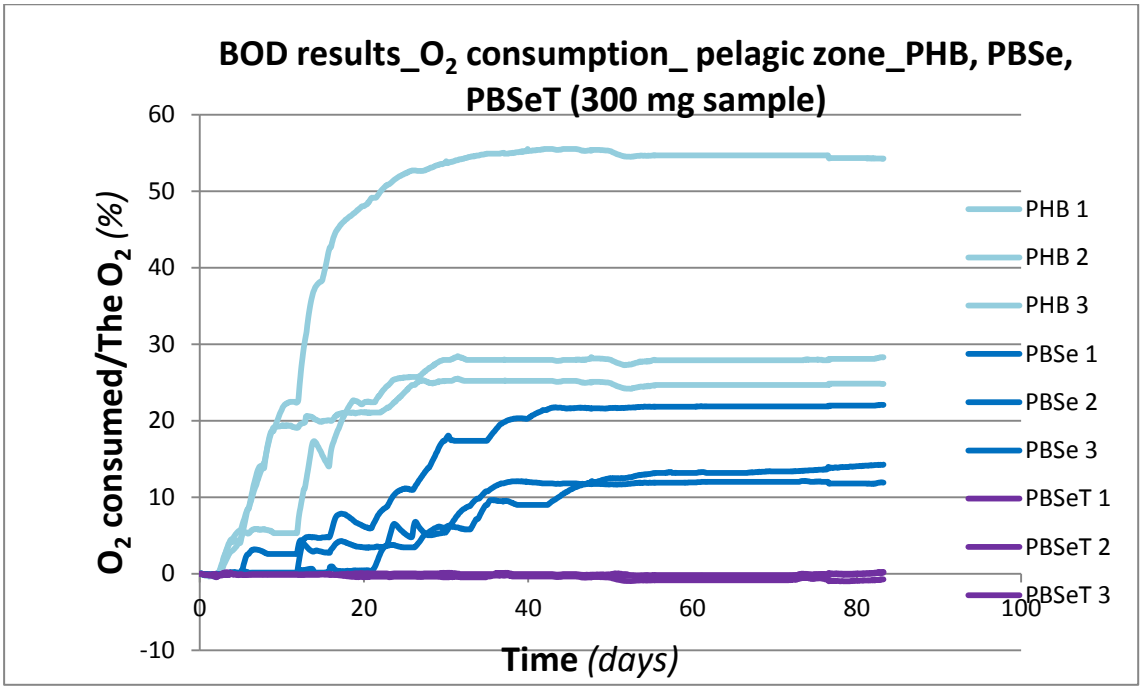


Figure 81. BOD results (O_2 consumption) biodegradation (%) vs. time for the pelagic lab test (300 mg sample)

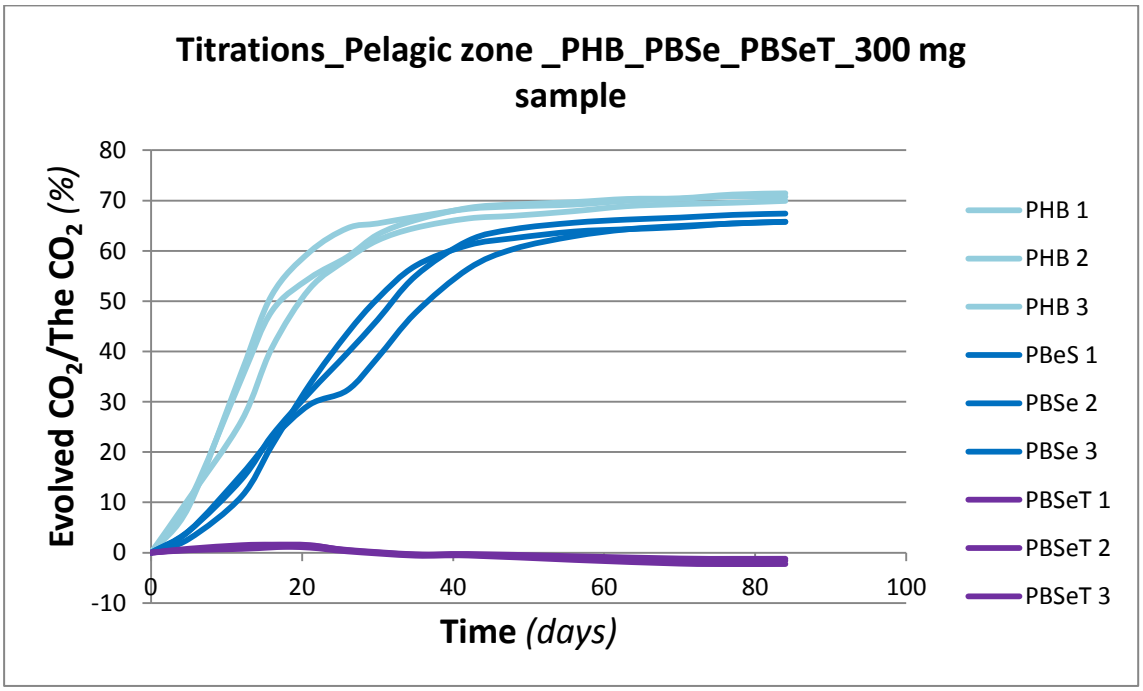


Figure 82. Titrations results biodegradation (%) vs. time for the pelagic lab test (300 mg sample)

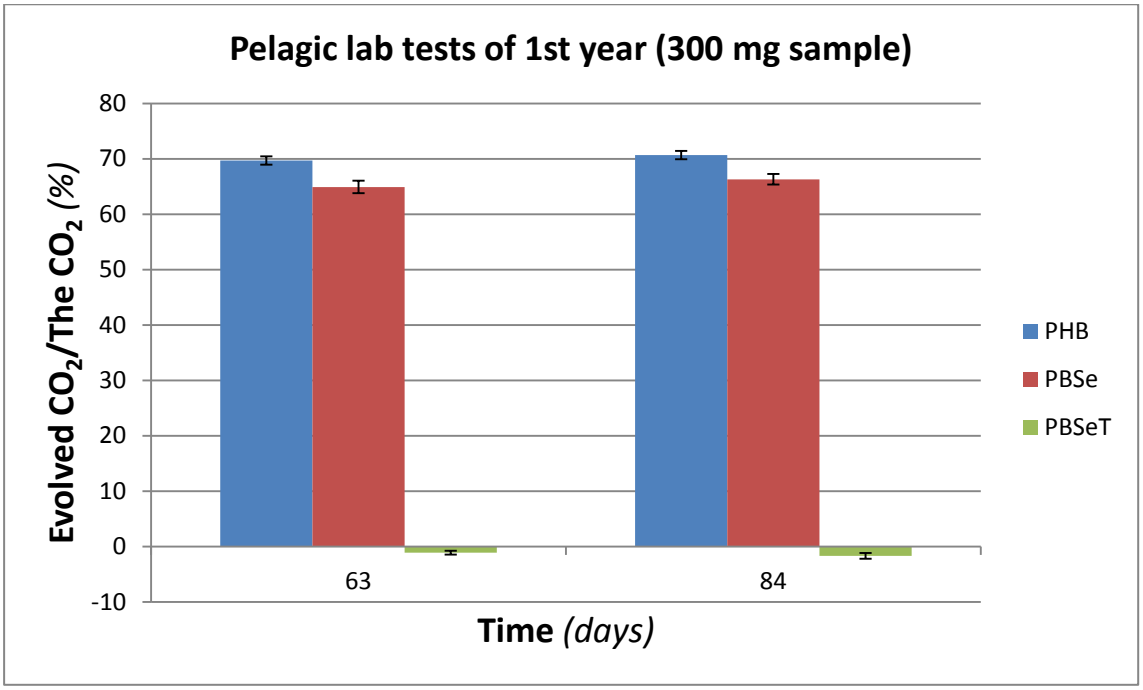


Figure 83. Titrations results biodegradation (%) vs. time after 63 and 84 days for the pelagic lab test (300 mg sample)

Table 27. Titrations results average biodegradation (%) and standard deviations after 84 days for the pelagic lab test (300 mg sample)

	PHB	PBSe	PBSeT
Average biodegradation (%)	70.7±0.7	66.3±0.9	-1.7±0.5

The biodegradation rates after 84 days are summarized in Table 27. The biodegradation follows the order PHB>PBSe>PBSeT. The fact that the oxygen consumption results were unreliable while for the biodegradation rates by means of CO_2 production were low it was decided to further examine the optimum amount of sample weight.

PHB 100 mg sample, PHB, cellulose 300 mg sample

The following experiment performed during the 1st year examined the biodegradation in the pelagic environment of two materials (PHB and cellulose), using 100 and 300 mg of sample for the PHB material and 300 mg of sample for the cellulose. The replicates included 3 blanks, 3 PHB samples (100 mg of each sample), 3 cellulose samples (300 mg per sample) and 3 PHB samples (300 mg per sample). The exact weights of samples and the corresponding organic carbon content of samples are presented in Table 28. The biodegradation values were determined by both continuous pressure measurements determining the oxygen consumption and titrations measuring CO_2 production.

Table 28. Weights and corresponding organic carbon of materials

Material	Weight of sample (mg)	Organic carbon in sample (g)
PHB 100 (1)	109.0	0.0521
PHB 100 (2)	104.4	0.0499
PHB 100 (3)	102.8	0.0492
cellulose 1	324.7	0.1442
cellulose 2	322.3	0.1431
cellulose 3	322.0	0.1430
PHB 300 (1)	306.9	0.1468
PHB 300 (2)	308.7	0.1476
PHB 300 (3)	306.5	0.1466

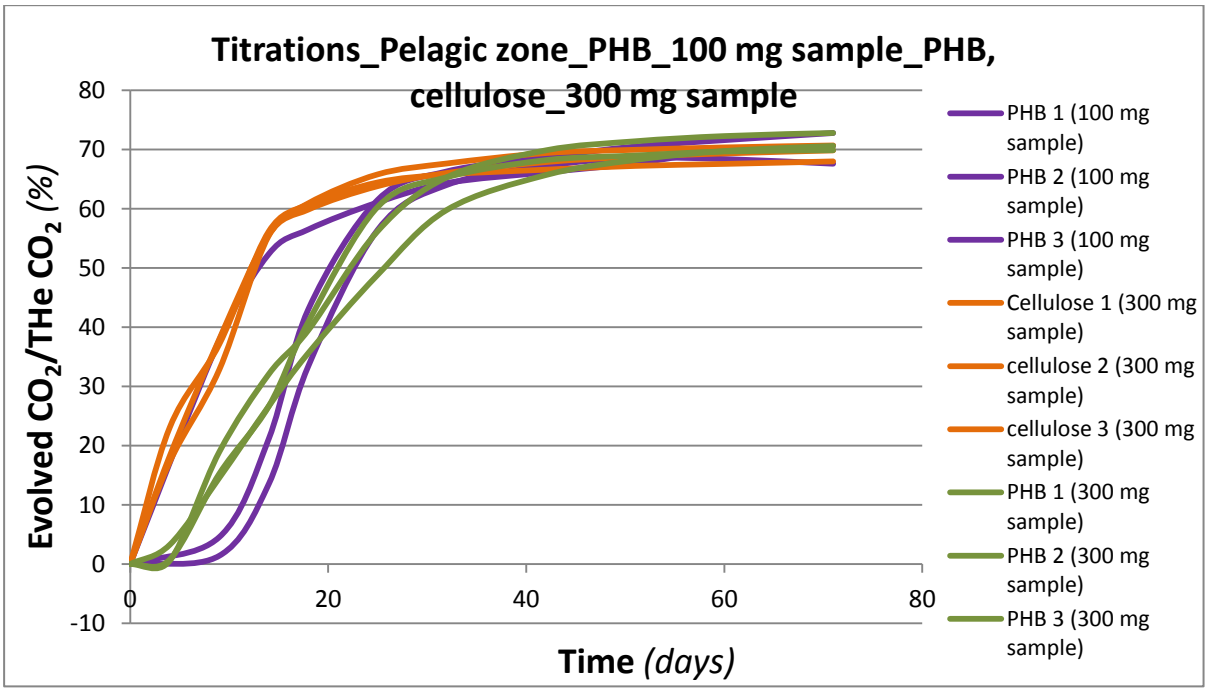


Figure 84. Titrations results biodegradation (%) vs. time for the pelagic lab test (100 and 300 mg sample)

Table 29. Titrations results_average % biodegradation after 71 days for the pelagic lab test (100 and 300 mg sample)

	PHB (100 mg)	Cellulose	PHB (300 mg)
Average CO_2 production (mg)	70.3±2.6	69.5±1.4	71.1±1.5

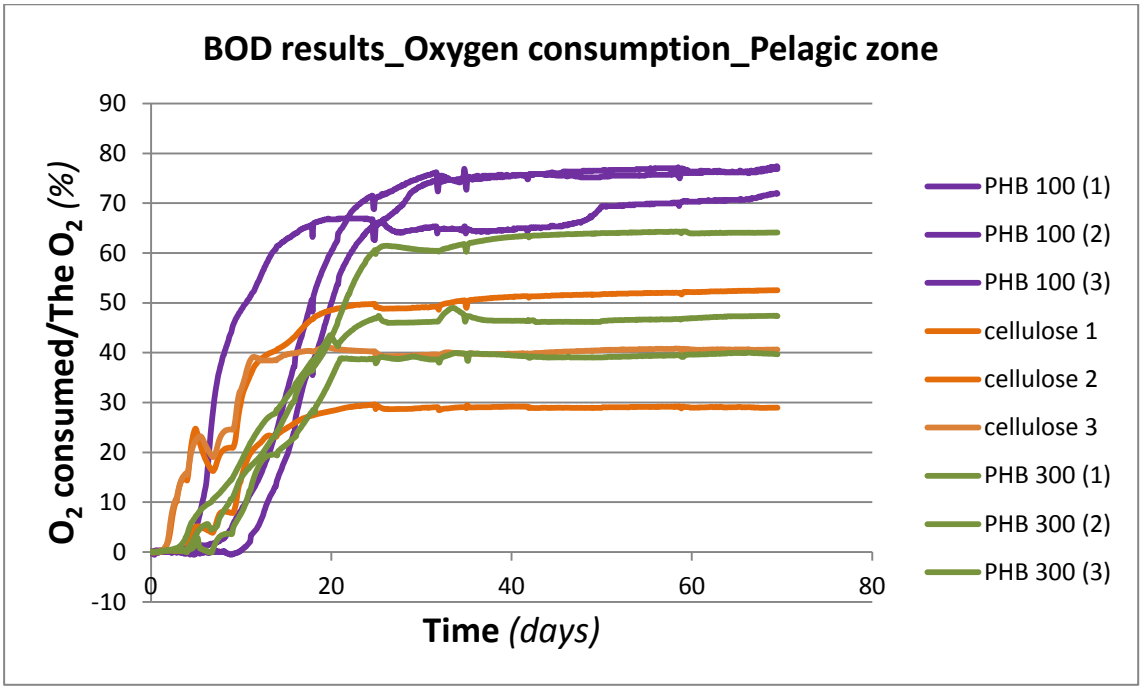


Figure 85. BOD results (O_2 consumption) biodegradation (%) vs. time for the pelagic lab test (100 and 300 mg sample)

Table 30. BOD results (O_2 consumption) average biodegradation (%) and standard deviations after 70 days for the pelagic lab test (100 and 300 mg sample)

	PHB (100 mg)	Cellulose	PHB (300 mg)
O_2 consumption average	75.3±3.0	40.7±11.8	50.4±12.4

The results from the CO_2 production determination were more reliable (significantly lower standard deviations values) than those from the O_2 consumption determination. Based on the former results the biodegradation reached a plateau when the degree was approximately 70% for all examined materials. Comparing the PHB of 100 mg samples and the PHB 300 mg samples the results were better for the PHB 300 mg samples (higher biodegradation values and lower standard deviations values). Based on these results it was chosen the amount of 300 mg for the samples as optimum quantity of samples.

PHB 300 mg sample (C/N test)

The following experiment performed for the 1st year of pelagic lab tests examined the effect of the amount of fertilizer in the pelagic environment. Samples of PHB of approximately 300 mg were used. Three different ratios of fertilizer "F2" corresponding to two replicates of PHB each were examined. The ratios used were: 1/5: N/C, 1/10: N/C and 1/20: N/C. The exact weights of samples and the corresponding organic carbon content of samples are presented in Table 31. For the experiment the biodegradation rates based on continuous

pressure measurements determining the oxygen consumption and the biodegradation values from CO_2 production based on titrations were determined.

Table 31. Weights and corresponding organic carbon of PHB

Material	Weight of sample (mg)	Organic carbon in sample (g)
PHB 1	305.7	0.1462
PHB 2	301.0	0.1439
PHB 3	307.3	0.1470
PHB 4	307.8	0.1472
PHB 5	305.5	0.1461
PHB 6	306.3	0.1465

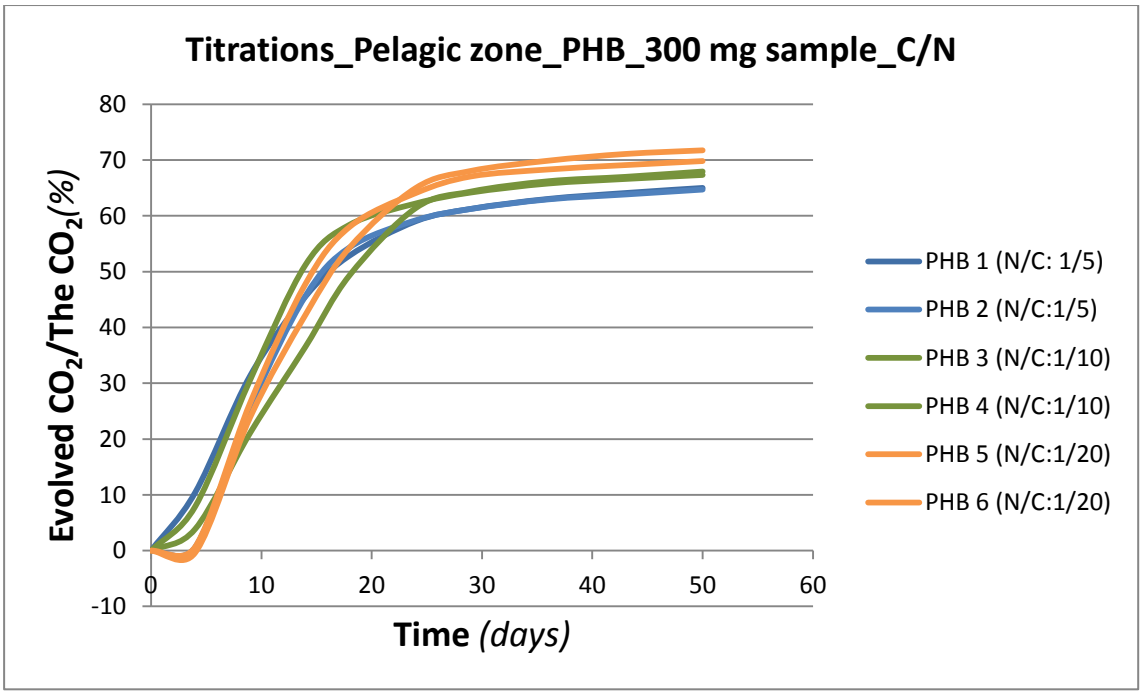


Figure 86. Titrations results biodegradation (%) vs. time for the pelagic lab test (300 mg sample, C/N different)

Table 32. Titrations results biodegradation (%) after 50 days for the pelagic lab test (300 mg sample, C/N different)

	N/C: 1/5	N/C: 1/10	N/C: 1/20
Average CO_2 production	64.9±0.2	67.6±0.4	70.8±1.3

Similarly to the previous experiments the results between the two methods for the determination of biodegradation presented discrepancies. The results from the CO_2 production determination were more reliable (significantly lower standard deviations values)

than those from the O_2 consumption determination. Based on these results the optimum nitrogen to organic carbon ratio was confirmed to be the N/C: 1/10 as it presented a slightly lower standard deviation value as compared to that of the ratio N/C: 1/20 and higher rate of biodegradation than that of the ratio N/C: 1/5.

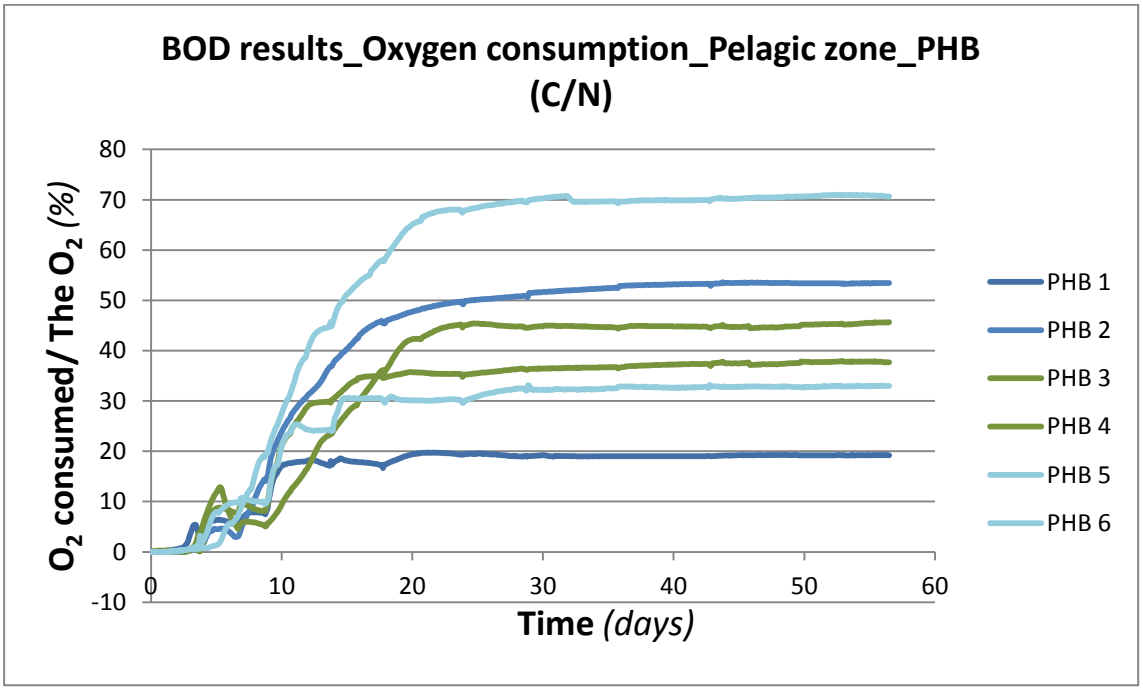


Figure 87. BOD results (O_2 consumption) biodegradation (%) vs. time for the pelagic lab test (300 mg sample, C/N different, PHB 1 and 2 have 1:5 C/N; PHB 3 and 4 have 1:10 C/N; PHB 5 and 6 have 1:20 C/N).

PHB 300 mg sample comparison of two methods (oxygen consumption vs. CO_2 production from titration method)

An experiment was executed to evaluate the difference between the two methods used for the determination of biodegradation (O_2 consumption determination by measuring constantly the pressures of the reactors and CO_2 production by titrating the trapping solutions inside the reactors). 3 PHB samples of approximately the same weight (Table 33) were used. In this experiment the reactors were aerated frequently (for the two first weeks almost twice per day).

Table 33. Weights and corresponding organic carbon of PHB samples (300 mg)

Material	Weight of sample (mg)	Organic carbon in sample (g)
PHB 300 (1)	297.7	0.1424
PHB 300 (2)	298.8	0.1430
PHB 300 (3)	297.3	0.1422

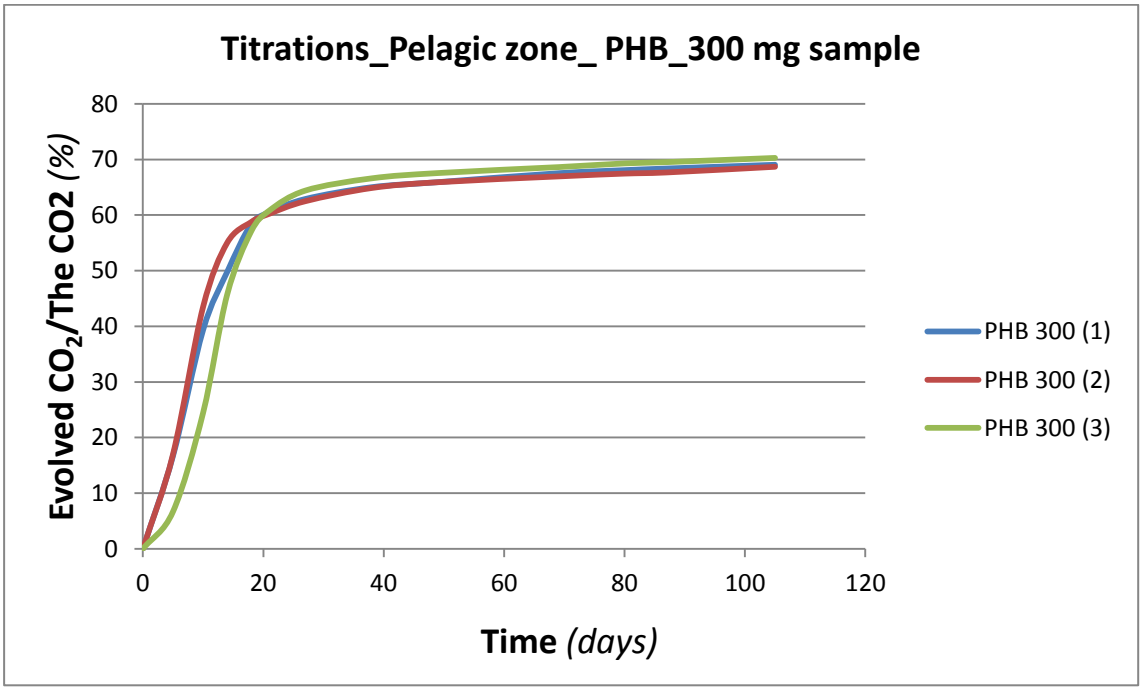


Figure 88. Titrations results biodegradation (%) vs. time for the pelagic lab test (300 mg of PHB samples)

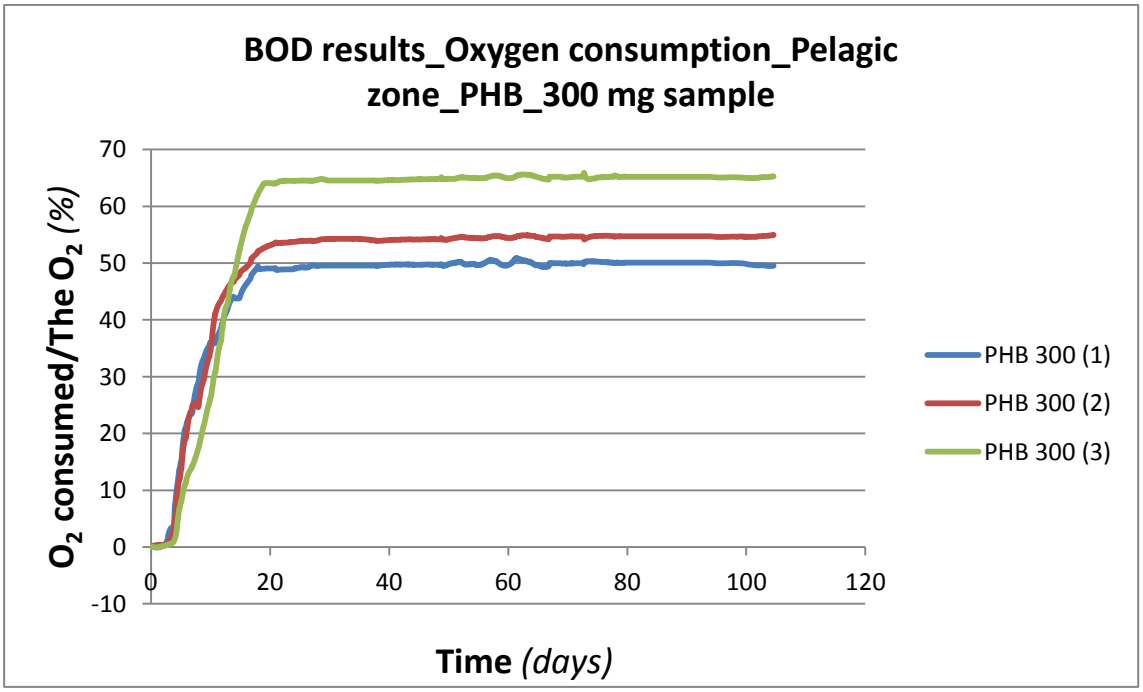


Figure 89. BOD results (O_2 consumption) biodegradation (%) vs. time for the pelagic lab test (300 mg of PHB samples)

For the three samples the biodegradation values for 105 days were determined by the two methods. At the end of the experiment after 105 days the results obtained are presented in Table 34.

Table 34. BOD results and titrations results after 105 days (O_2 consumption and CO_2 production) for PHB

	O_2 consumption	CO_2 production
Average	56.6±8.0	69.3±0.8

As it can be concluded from Table 34 the rates of biodegradation are higher and the standard deviation values are lower for the CO_2 production method.

Closed vessel system

The effect of the reactor volume was examined for the pelagic lab tests. Instead of using the 250 ml flasks, glass flasks of 4L volume were used for this experiment. The reactor was filled with 100 ml seawater and PHB film of 300 mg was placed in the reactor. In the blank reactor no material was introduced. The examined system constituted one blank and one PHB. Nutrients were added to both reactors by adding “Haifa” fertilizer so to correspond to a ratio of N/C: 1/10. Stirring bars were added to both reactors which were placed on magnetic stirrers. Trapping solutions for the CO_2 produced were KOH solutions of 1N and the traps were placed inside the reactors above the seawater. The method used was based on the CO_2 determination by titration with HCl 0.25N. The parameters of the experimental set-up are summarized in Table 35.

Table 35. Parameters of the experimental set-up of the pelagic lab test (Closed vessel)

Reactor volume (L)	4
Type	Static with stirring
Temperature (°C)	25-28
Sample characteristics	Plastic specimen
Quantity of sample (mg)	300
Quantity of inoculum (ml) (natural seawater)	100
Measurement method	CO_2 titration
Chemical reagent	KOH 1 M and HCl 0.25 M
Nutrients	Haifa fertilizer 1/10 (N/C)

As it is shown in Figure 90, the biodegradation rate reached at this experiment was approximately 95% after 127 days, but the plateau phase was not still reached. This value is significantly higher than 70% value that is reached when using the 250 ml reactors. The biodegradative trends given in Figure 89 and Figure 90 were different. In the first case the plateau was totally reached in approx. 20 days but in the second case, after the same period of time, there was a clear change of slope but the plateau was not reached.

These very preliminary results suggest that the oxygen available in the reactor could affects the biodegradation which could be limited for a smaller available volume of reactor under pelagic conditions. Other investigations are needed to confirm this fact.

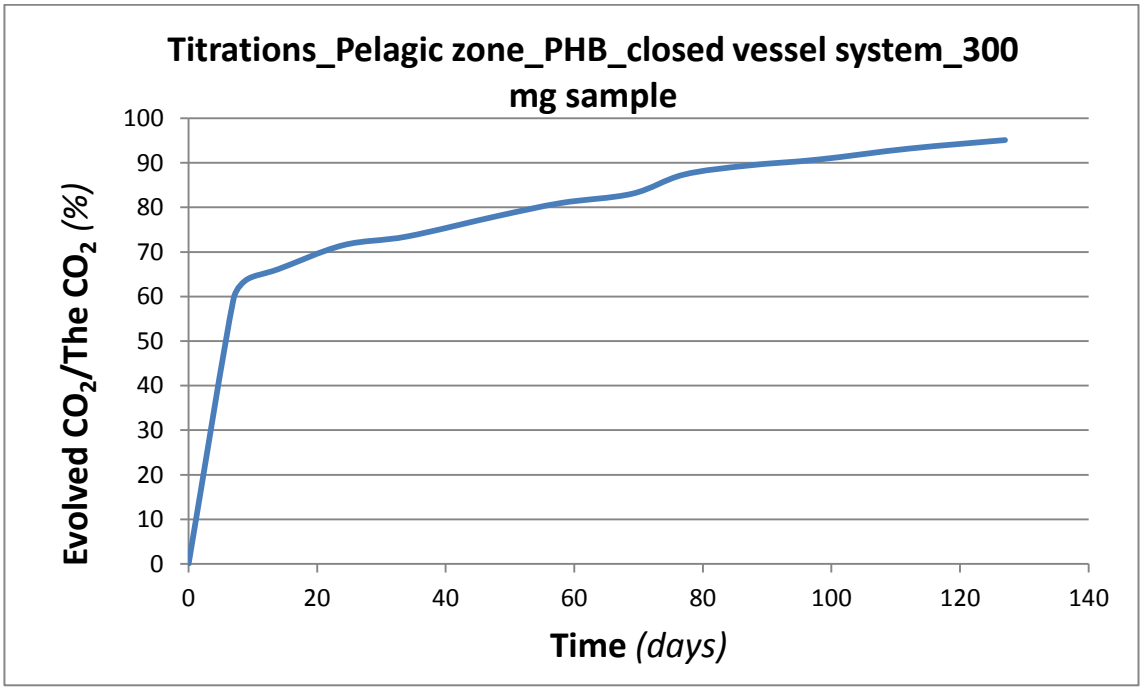


Figure 90. Titrations results biodegradation (%) vs. time for the pelagic lab test (300 mg of sample, clossed vessel)

The system based on the determination of CO_2 production had good results but a discrepancy was observed between the biodegradation results based on the titrations and the biodegradation results produced from oxygen consumption based on the pressures recorded.

5.5 Laboratory: BASF

BASF has, in all tests, used Oxytop set-up. In Table 36 the biodegradation of all marine environments results obtained are reported. BASF participated to the biodegradation test using only the Italian inoculum.

Table 36. Biodegradation results (%) obtained using the Oxitop system

Italian inoculum	days	Sample	Biodegradation (%)
Eulittoral	50	Blank	/
		LDPE	1.1 ± 0.2
		PBSe	2.9 ± 2.2
		PBSeT	2.5 ± 0.6
		PHB	2.4 ± 1.0

		Filter paper	2.8 ± 1.9
	100	**Blank**	/
		LDPE	1.7 ± 2.0
		PBSe	3.2 ± 4.9
		PBSeT	3.3 ± 3.5
		PHB	5.3 ± 0.6
		Filter paper	13.3 ± 3.5
	150	**Blank**	/
		LDPE	-0.2 ± 3.2
		PBSe	12.2 ± 13.2
		PBSeT	7.5 ± 4.3
		PHB	16.8 ± 4.6
		Filter paper	21.1 ± 3.8
	220	**Blank**	/
		LDPE	0.9 ± 3.4
		PBSe	27.4 ± 26.3
		PBSeT	26.0 ± 19.0
		PHB	52.3 ± 18.1
		Filter paper	33.7 ± 9.7
Benthic	**60**	**Blank**	/
		LDPE	0.5 ± 0.9
		PBSe	7.2 ± 0.8
		PBSeT	5.9 ± 0.4
		PHB	9.1 ± 3.7
		Filter paper	5.8 ± 2.1
	115	**Blank**	/
		LDPE	0.8 ± 2.1
		PBSe	38.5 ± 2.9
		PBSeT	32.7 ± 2.8
		PHB	53.2 ± 23.2
		Filter paper	23.2 ± 3.1
	175	**Blank**	/
		LDPE	2.3 ± 2.4
		PBSe	79.8 ± 5.2
		PBSeT	61.4 ± 3.8
		PHB	72.4 ± 19.0
		Filter paper	56.3 ± 5.3
	280	**Blank**	/
		LDPE	2.3 ± 3.5 (261 days)
		PBSe	89.0 ± 21.0 (261 days)

		PBSeT	81.1 ± 1.7
		PHB	87.6 ± 0.3
		Filter paper	91.6 ± 4.9
Pelagic	**50**	**Blank**	/
		LDPE	0.3 ± 0.8
		PBSe	12.4 ± 6.9
		PBSeT	28.6 ± 33.9
		PHB	35.2 ± 26.5
		Filter paper	4.0 ± 3.3
	100	**Blank**	/
		LDPE	0.8 ± 1.1
		PBSe	20.3 ± 10.7
		PBSeT	40.5 ± 39.2
		PHB	52.3 ± 22.8
		Filter paper	5.3 ± 4.6
	150	**Blank**	/
		LDPE	1.3 ± 1.2
		PBSe	23.7 ± 11.1
		PBSeT	48.9 ± 43.0
		PHB	66.7 ± 13.8
		Filter paper	5.6 ± 4.8 (126 days)
	200	**Blank**	/
		LDPE	2.1 ± 1.2
		PBSe	26.0 ± 11.6
		PBSeT	52.9 ± 46.9
		PHB	75.2 ± 8.5
		Filter paper	/

A general observation should be done, in some cases a very high speed of biodegradation was recorded and in this moment a black spot appears due to the O_2 depletion (PHB test materials, benthic environment). Also methane was measured that indicated that anaerobic conditions appeared causing the obtaining of not completely reliable biodegradation measure.

6 Laboratory results of year 2

The biodegradation tests in the three marine environments were repeated for the second year following the method modification suggestions obtained after the general discussion occurred during the project meeting. The sediments and the seawater were collected in the same places of year 1 (Elba Island and Salamina Island) and in the same period (summer). The chemical characterization of the seawater and sediments was performed by Open-Bio partner ISA. In Table 37 are reported the results.

Table 37. Chemical characterization of the marine inoculum used to perform the second year of marine biodegradation laboratory experiments.

SEAWATERS									
Sample	**TC (mg/l)**	**IC (mg/l)**	**OC (mg/l)**			**nitrite (mg/l)**	**nitrate (mg/l)**	**phosphate (mg/l)**	**ammonium (mg/l)**
Natural seawater (Elba)	41	14	27			< 0-10	1.14	< 1	64.1
Natural seawater (Salamina)	39	17	22			< 0-10	0.47	< 1	88.5
SEDIMENTS									
	C (%)	**IC (%)**	**OC (%)**	**N (%)**	**H (%)**	**nitrite (ppm)**	**nitrate (ppm)**	**phosphate (ppm)**	**ammonium (ppm)**
Eulittoral sediment (Elba)	0.11	<0.10	**< 0.10**	0.00	5.22	< 0.003	2.1	0.48	38
Eulittoral sediment (Salamina)	7.32	6.06	**1.26**	0.01	3.93	< 0.003	0.083	0.5	20
Benthic sediment (Salamina)	8.63	5.05	**3.58**	0.05	2.21	< 0.003	9	0.4	8
Benthic sediments (Elba)	6.03	5.15	**0.88**	0.04	5.79	< 0.003	0.014	0.38	<5

The two seawaters sampled in two different locations seem similar, but the sediments from Salamina Island show an high amount of organic carbon when compared with those collected in Elba Island.

6.1 Laboratory: Novamont

6.1.1 Biodegradation in Eulittoral zone

The experiment was repeated in the same conditions of the first year with a sediment collected in Italy and in Greece respectively in July and September 2015. In this second year of test the samples were tested in form of film and in form of powder to improve the contact with the sediment and to promote the microbial attack. The second improvement was the

addition of nutrients in the sediment; namely nitrogen in nitric form as $NaNO_3$ in a quantity of 0.1g N/ 1g of TOC.

Both tests (with Italian and Greek inoculum) were run for more than 250 days and the biodegradation results were reported in Figure 91 and Figure 92. Table 38 and Table 39 show the CO_2 cumulative production and the biodegradation level achieved by the test materials. Also the standard deviations are reported.

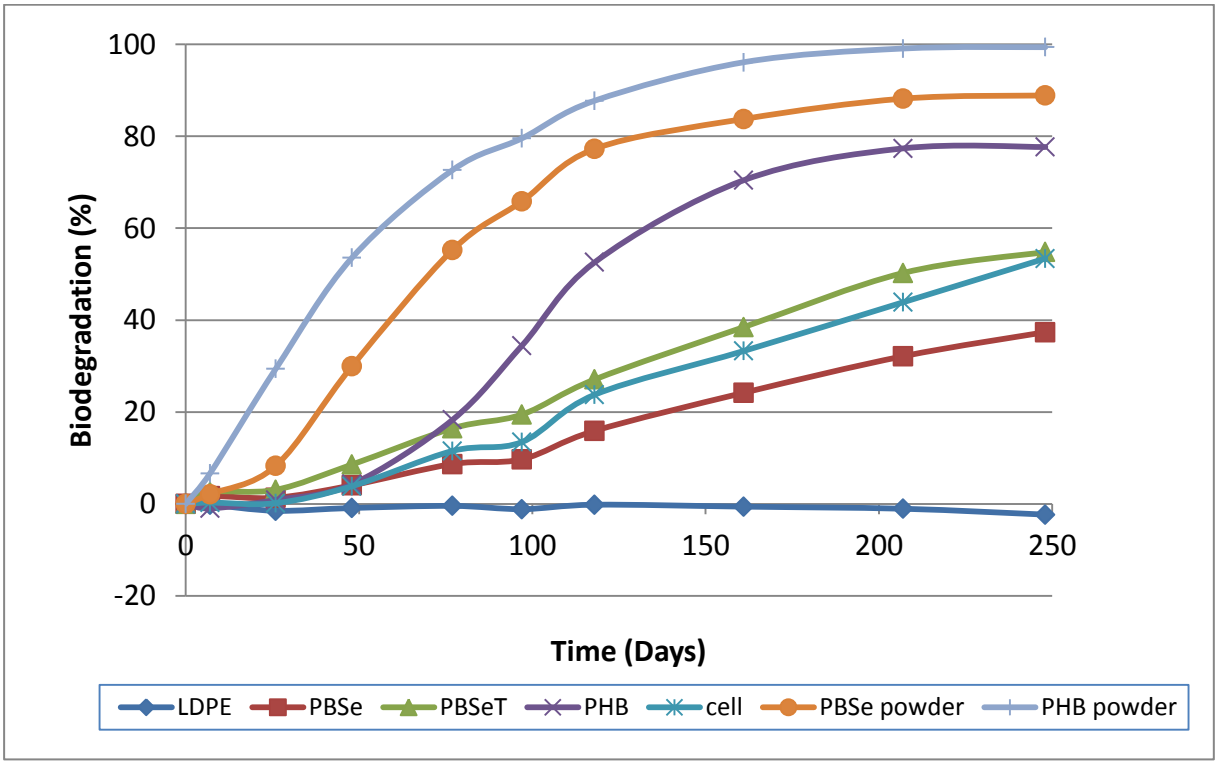


Figure 91. Biodegradation in Eulittoral environment Italian sediment

From the biodegradation trend is seen that all test materials were in biodegradation phase and that PHB drives the test with a higher biodegradation level achieved. The use of powder speeds up the biodegradation of the PHB and PBSe. Also the standard deviation is lower than the ones obtained using the same test material in form of film. The addition of nutrients does not seem to have a clear effect. Generally this appears a promising test but the use of powdered test material can help to reduce the standard deviation. The use of test specimen in form of film can help to better understand if a low (or high) biodegradation is effectively correct by a simple visual inspection inside the reactor i.e a very high standard deviation was obtained for PHB film and cellulose filter. Material residual was collected in the reactor 12 and the cellulose was effectively only partially biodegraded confirming the low biodegradation registered.

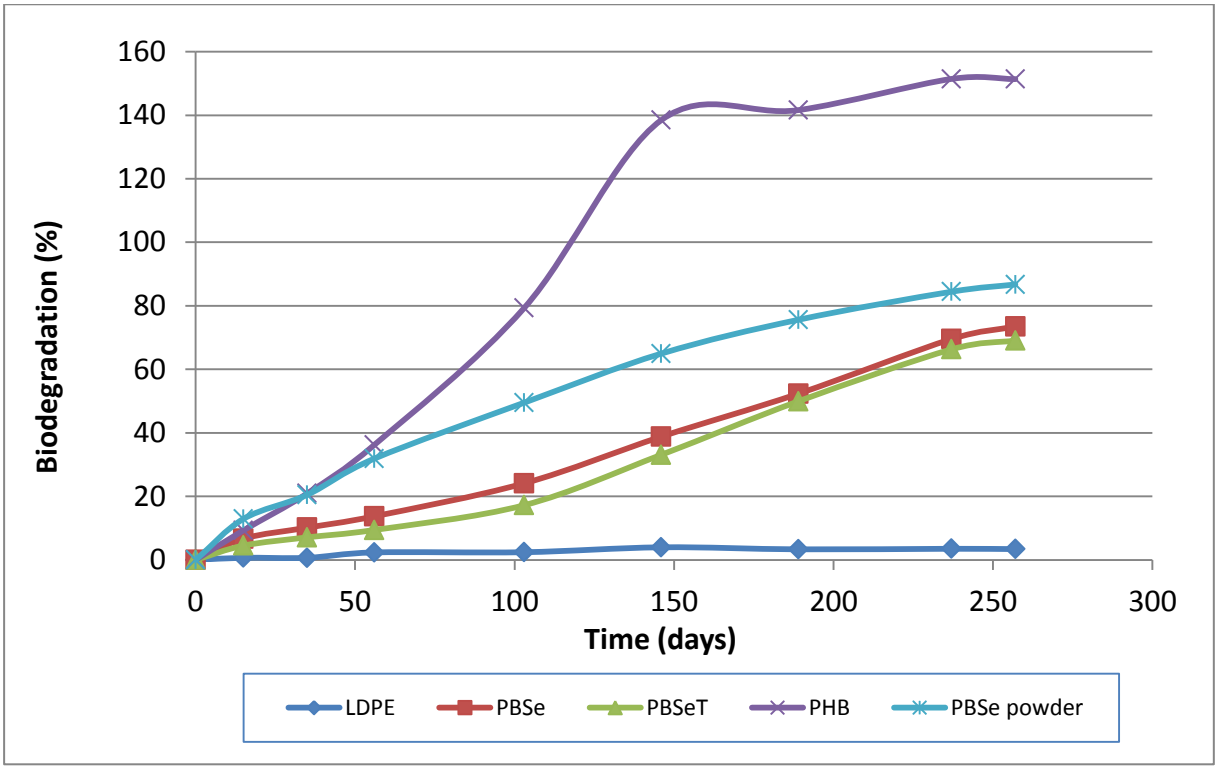


Figure 92. Biodegradation in Eulittoral environment Greek sediment

Table 38. Biodegradation in Eulittoral environment after 248 days (Italian sediment)

Reactor number	Test materials	CO_2 cumulative Production (mg)	Biodegradation (%)	Biodegradation averages
1	Control	209.22	-	
2	Control	140.84	-	
3	LDPE (negative control)	181.10	1.92	-2.32% (±6)
4	LDPE (negative control)	154.31	-6.55	
5	PBSe	250.40	31.03	37.38% (±9)
6	PBSe	278.92	43.72	
7	PBSeT	316.8	58.95	54.81% (±5.9)
8	PBSeT	296.08	50.66	
9	PHB	359.964	103.68	77.66%(±36.8)
10	PHB	265.98	51.64	
11	Cellulose Filter paper	334.224	100.98	77.19% (±33.6)
12	Cellulose Filter paper	256.872	53.40	
13	PBSe powder	380.16	85.04	88.87% (±5.4)
14	PBSe powder	396.66	92.70	
15	PHB powder	336.864	90.65	99.38 (±12.3)
16	PHB powder	368.94	108.10	

The blank average production (only sediment without test material) was about 182 mg of CO_2 after 253 days in the first year of test, while in the second year after 248 days the CO_2 production was very similar 175 mg. Also the biodegradation of the different test materials was similar, the problem is the standard deviation, the use of powder can help.

Table 39. Biodegradation in Eulittoral environment after 257 days (Greek sediment)

Reactor number	Test materials	CO_2 cumulative production (mg)	Biodegradation (%)	Biodegradation Averages
1	Control	113.52	-	
2	Control	149.95	-	
3	LDPE	144.14	3.96	3.41% (±0.78)
4	LDPE	140.45	2.86	
5	PBSe	215.03	34.22	**73.49% (±55.54)**
6	PBSe	397.85	112.77*	
7	PBSeT	278.65	60.29	69.07% (±12.42)
8	PBSeT	312.84	77.85	
9	PHB	395.01	150.69*	**151.36%(±0.96)**
10	PHB	401.28	152.04*	
11	PBSe powder	311.00	73.82	86.73% (±18.26)
12	PBSe powder	376.86	99.64	

*: unrealistic values

The same test performed using as inoculum the sand coming from Greece gave for PHB overestimation of CO_2 (and consequently its biodegradation) and at the same way an overestimation of CO_2 coming from the reactor 6 (PBSe test material). This behaviour determined the very high standard deviation of PBSe specimen for the materials tested in form of film. Also in this case the visual inspection of R5 confirmed that the PBSe film specimen was only partially biodegraded. The same test material (PBSe) tested in powder form highlighted a more quick biodegradation joined to a lower standard deviation.

6.1.2 Biodegradation in Benthic zone

The experiments were repeated in the same conditions of the first year but applying a dilution of the sediment in order to decrease the background production of CO_2. A sediment collected in Italy and in Greece respectively in July and September 2015 was used. As highlighted in the first year, the sediment in the Benthic zone collected at 30/40m of depth, had an high organic content and a potential high CO_2 production. During the meeting in Gent it was decided to treat the inoculum before starting the test. In particular in a first case the sediment was diluted with the same sediment after an incineration at 550°C (in order to eliminate the organic fraction) and in a second case air was fluxed in the sediment during the preliminary phase (without the samples) in order to promote the biodegradation of the organic matter contained in the sediment and to decrease its background activity. In this year

the tests performed using the Italian inoculum were performed at 25°C instead 28°C. The conditions and the methodology adopted were the same used in the first year.

Figure 93 and Table 40 report the results obtained using the Italian inoculum after a dilution with calcined inoculum (33:67 w.w.). The biodegradation trends highlighted a very clear biodegradation: LDPE did not show any biodegradation and all test materials are biodegraded at the same time with the same speed. PBSeT, PHB and Cellulose paper reached 100% biodegradation and PBSe 95%. Standard deviations are clearly lower than during the first year experiment.

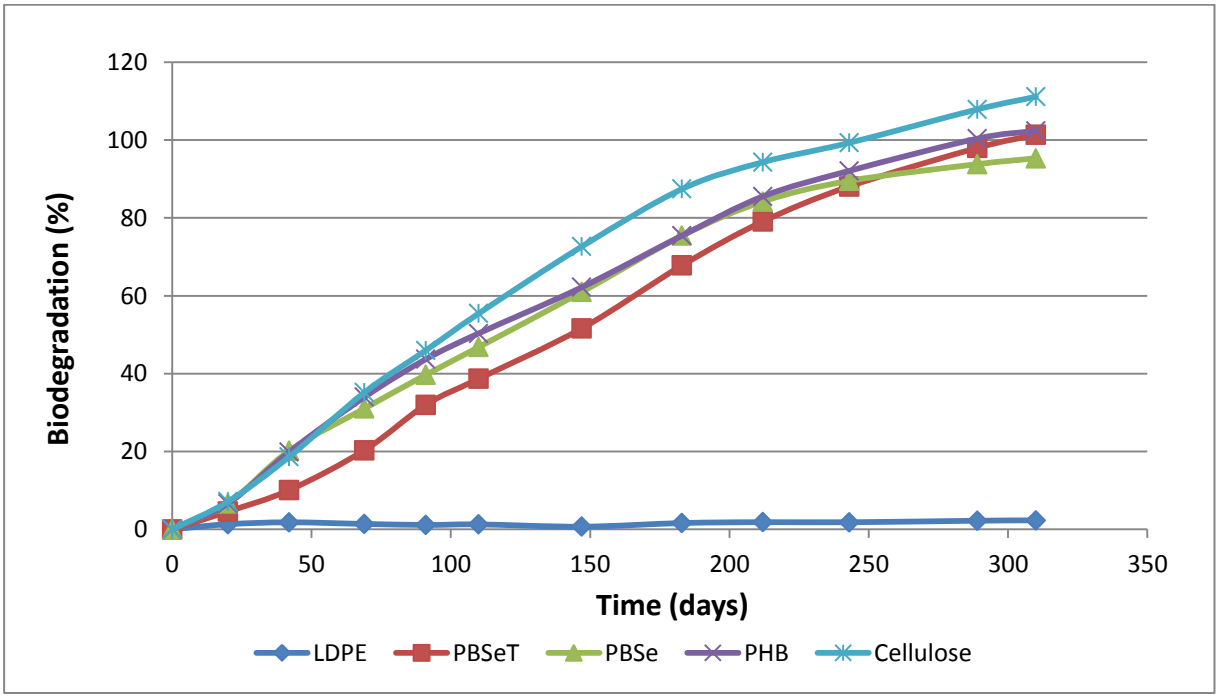


Figure 93. Biodegradation trends in benthic environments (Italian inoculum) after dilution of sand inoculum with calcined inoculum (33:67 w.w.). Test performed at 25°C

Table 40. CO_2 production and biodegradation achieved by each reactor and biodegradation average plus standard deviation

Reactor number	Test material	CO_2 cumulative production (mg)	Biodegradation (%)	Biodegradation averages (%)
1	Control	28.42	-	-
2	Control	27.21	-	
3	Control	31.11	-	
4	LDPE	30.20	2.47	2.31 (±1.79)
5	LDPE	29.13	0.44	
6	LDPE	30.80	4.01	
7	PBSeT	74.41	104.54	101.38 (±5.86)
8	PBSeT	71.46	104.99	

9	PBSeT	75.32	94.62	
10	PBSe	70.59	109.97	95.33 (±13.43)
11	PBSe	56.97	83.58	
12	PBSe	65.88	92.46	
13	PHB	63.66	96.49	102.39 (±9.04)
14	PHB	58.30	112.80	
15	PHB	59.93	97.89	
16	Cellulose	60.09	111.14	111.14

At the same time a second test was performed using the Italian inoculum submitted to an air flux and a gentle mixing (during 10 days) in order to promote the oxidation of labile organic matter before to prepare the reactors. In Figure 94 and Table 41 are reported the results obtained. This kind of pre-treatment is not sufficient to consume the excess of organic matter and that the CO_2 overproduction of LDPE is still well visible. Moreover, biodegradation values above 100% (± 130%) are reached for Cellulose and PHB and the biodegradation is still increasing at the end of the test. At the end of the test LDPE showed a not realistic biodegradation of 20%. LDPE test items were weighted and the weight demonstrated that no biodegradation occurred (the weights were identical to the weight at start of the test). Also the standard deviation values are relatively high when compared to the previous approach.

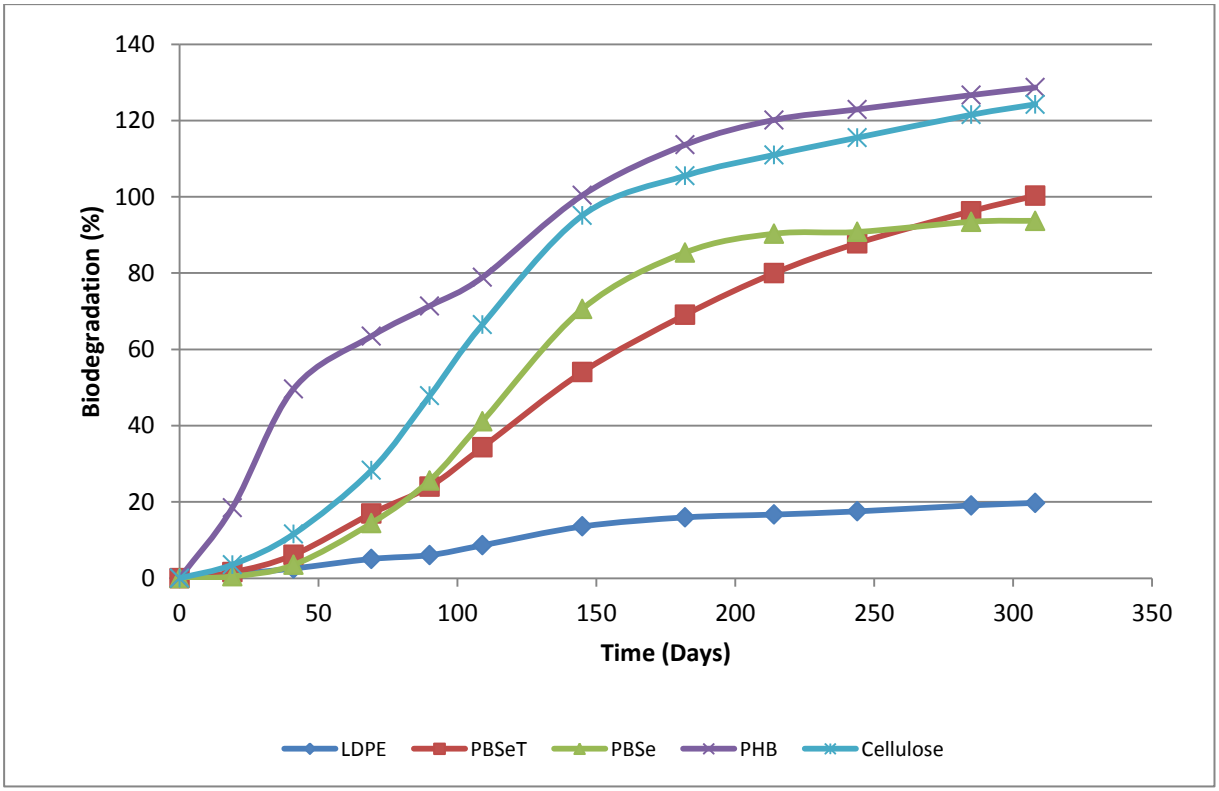


Figure 94. Biodegradation trend in benthic environments (Italian inoculum) after one week of air flush as inoculum pre-treatment. Test performed at 25°C.

Table 41. CO_2 production and biodegradation achieved by each reactor and biodegradation average plus standard deviation

Reactor number	Test material	CO_2 cumulative production (mg)	Biodegradation (%)	Biodegradation averages (%)
1	Control	73.30	-	-
2	Control	74.43	-	
3	Control	83.00	-	
4	LDPE	95.73	36.91	19.75 (±14.86)
5	LDPE	83.11	11.08	
6	LDPE	82.76	11.27	
7	PBSeT	111.96	107.76	100.29 (±6.51)
8	PBSeT	113.03	97.26	
9	PBSeT	110.93	95.85	
10	PBSe	105.01	81.12	93.71 (±18.26)
11	PBSe	117.48	114.65	
12	PBSe	113.67	85.36	
13	PHB	124.26	157.75	128.65 (±30.91)
14	PHB	105.52	96.20	
15	PHB	120.17	132.01	
16	Cellulose	114.47	136.36	124.25 (±16.14)
17	Cellulose	114.18	130.46	
18	Cellulose	109.86	105.93	

The same approach was adopted for the Greek sediment as for the Italian sediment. In this case the incubation was performed at 28°C due to laboratory equipment availability. In this specific case the calcination of sediment to perform the dilution process caused a not controlled increase of the pH values (more than 9) that determined the loss of biodegradation capacity of the sediment. In Table 42 and Figure 95 are reported the results. The graph highlighted that only a very minimal activity remains and the biodegradation speed was very low compared to the others tests. In this specific case also the Cellulose paper was not completely biodegraded during 250 days.

To complete the matrix of tests also for the Greek inoculum the air flux of the sediment was performed (10 days with gentle mixing). Results are shown in Figure 96 and Table 43. Also in this case the pre-treatment was not sufficient to consume the extra organic matter present in the sediment. The same behaviour was observed (overestimation of CO_2 and unreliable biodegradation) and a conspicuous standard deviation.

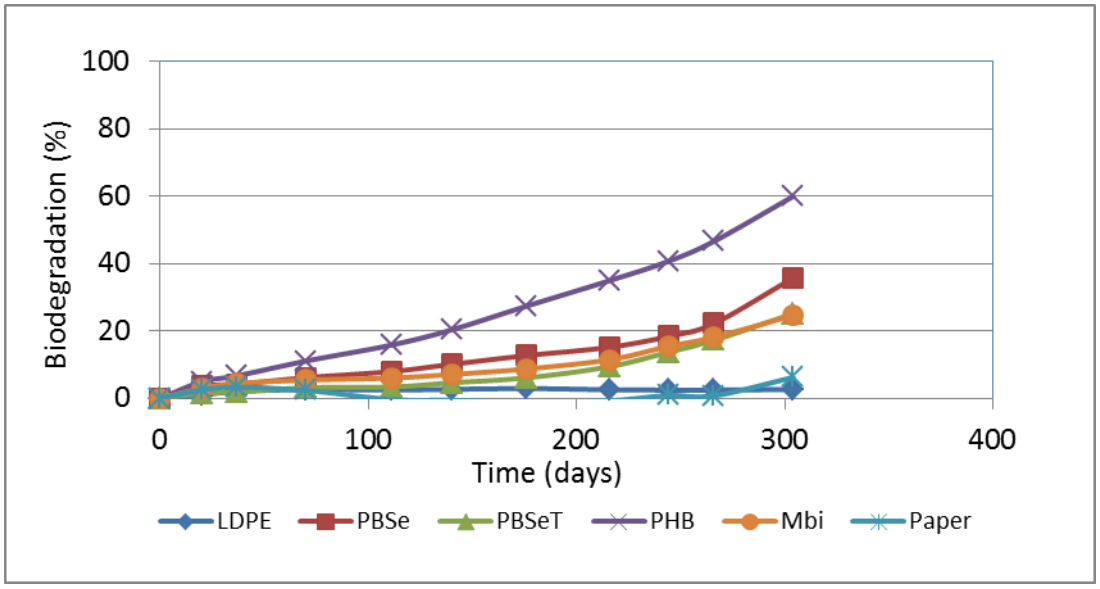


Figure 95. biodegradation trends in benthic environments (Greek inoculum) after dilution of sand inoculum with calcined inoculum (50:50 w.w.). Test performed at 28°C

Table 42. CO_2 production and biodegradation achieved by each reactor and biodegradation average plus standard deviation

Reactor number	Test material	CO_2 cumulative production (mg)	Biodegradation (%)	Biodegradation averages (%)
1	Control	27.76	-	-
2	Control	19.81	-	
3	Control	24.55	-	
4	LDPE	23.36	-1.02	2.62 (±3.61)
5	LDPE	28.04	6.20	
6	LDPE	25.65	2.68	
7	PBSe	41.33	37.11	35.76 (±3.87)
8	PBSe	41.01	38.78	
9	PBSe	38.02	31.40	
10	PBSeT	41.65	31.40	25.16 (±9.90)
11	PBSeT	35.31	36.25	
12	PBSeT	32.70	22.03	
13	PHB	40.93	45.61	60 (±39.37)
14	PHB	60.30	104.54	
15	PHB	34.66	29.84	
16	Cellulose	26.08	6.19	6.19

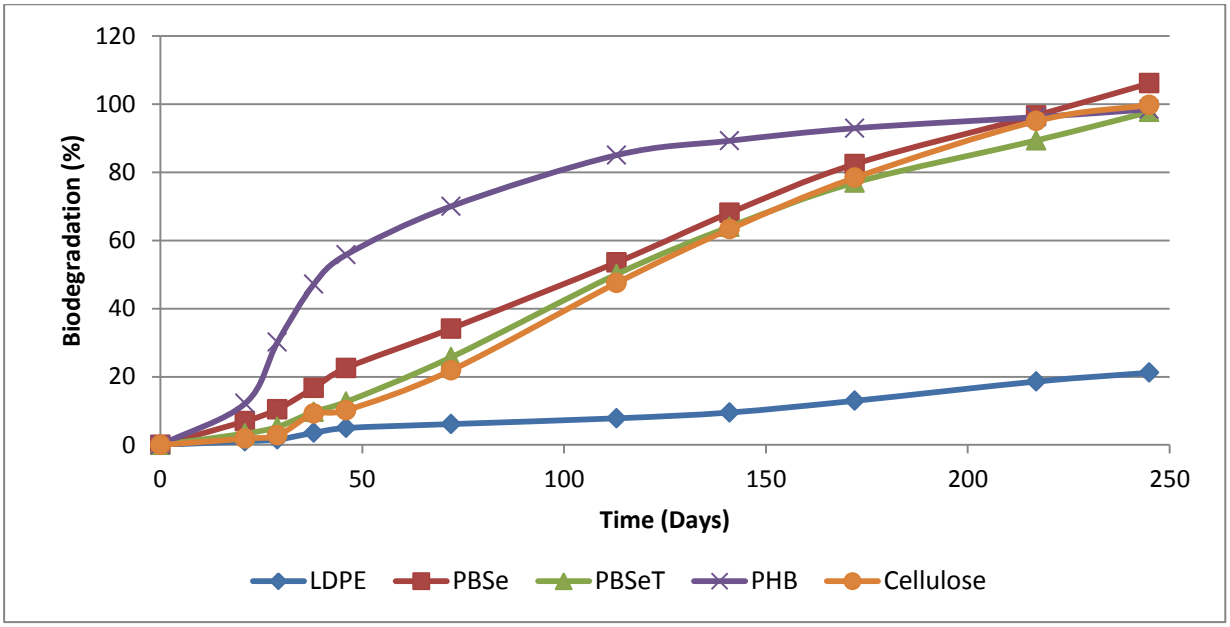


Figure 96. Biodegradation trends in benthic environments (Greek inoculum) after one week of air flush as inoculum pre-treatment. Test performed at 28°C.

Table 43. CO_2 production and biodegradation achieved by each reactor and biodegradation average plus standard deviation

Reactor number	Test material	CO_2 cumulative production (mg)	Biodegradation (%)	Biodegradation averages (%)
1	Control	60.94	-	-
2	Control	63.10	-	
3	Control	61.68	-	
4	LDPE	82.94	32.55	21.19 (±10.54)
5	LDPE	69.10	11.73	
6	LDPE	73.38	19.28	
7	PBSe	108.45	104.55	106.12 (±10.92)
8	PBSe	110.88	117.74	
9	PBSe	103.71	96.08	
10	PBSeT	98.75	80.74	97.70 (±24.85)
11	PBSeT	119.82	126.22	
12	PBSeT	103.32	86.14	
13	PHB	101.49	103.95	98.49 (±4.76)
14	PHB	98.13	96.37	
15	PHB	98.99	95.17	
16	Cellulose	94.10	99.79	99.79

6.1.3 Biodegradation in Pelagic zone

Before the start of the tests both seawaters were amended with KH_2PO_4 (0.1 g/l) and NH_4Cl (0.05 g/l) as performed during the first year. They serve as nutrients for the micro-organisms. Two test were performed: Biodegradation in Greek seawater with determination of O_2 consumed plus titration of KOH as double check (Oxitop system). Three replicates each test materials, three blank controls. Test temperature was 28°C (±2). The same test was repeated with the Italian seawater. The test items were milled to powders using liquid nitrogen. The reactors used were glass flasks of 250 ml. The flasks were filled with 82 ml of seawater with KH_2PO_4 (0.1 g/l) and NH_4Cl (0.05 g/l). About 20 mg of each test material (milled form) was put in the seawater (not applied for the control reactors). The Oxytop® system was used for determination of biodegradation measuring the oxygen consumption and trapping the CO_2 produced with a KOH solution (in this specific case 1 N) that it was possible to titrate during the test in order to have a double check of the biodegradation reactions: oxygen consumption and CO_2 development. Cellulose was tested only using the Italian seawater due to laboratory equipment availability.

Figure 97 shows the biodegradation trend obtained using the O_2 consumption measurements on the Greek seawater. LDPE did not show any biodegradation, on the contrary PHB and PBSe highlighted a high level of biodegradation achieved with a very high rate. At the same time two titrations of KOH trap were performed. The data obtained are in line with the biodegradation registered using the O_2 consumption. Data are shows in Figure 98. In Table 44 were reported the data of biodegradation calculated by the O_2 consumption and based the CO_2 production followed by their standard deviation.

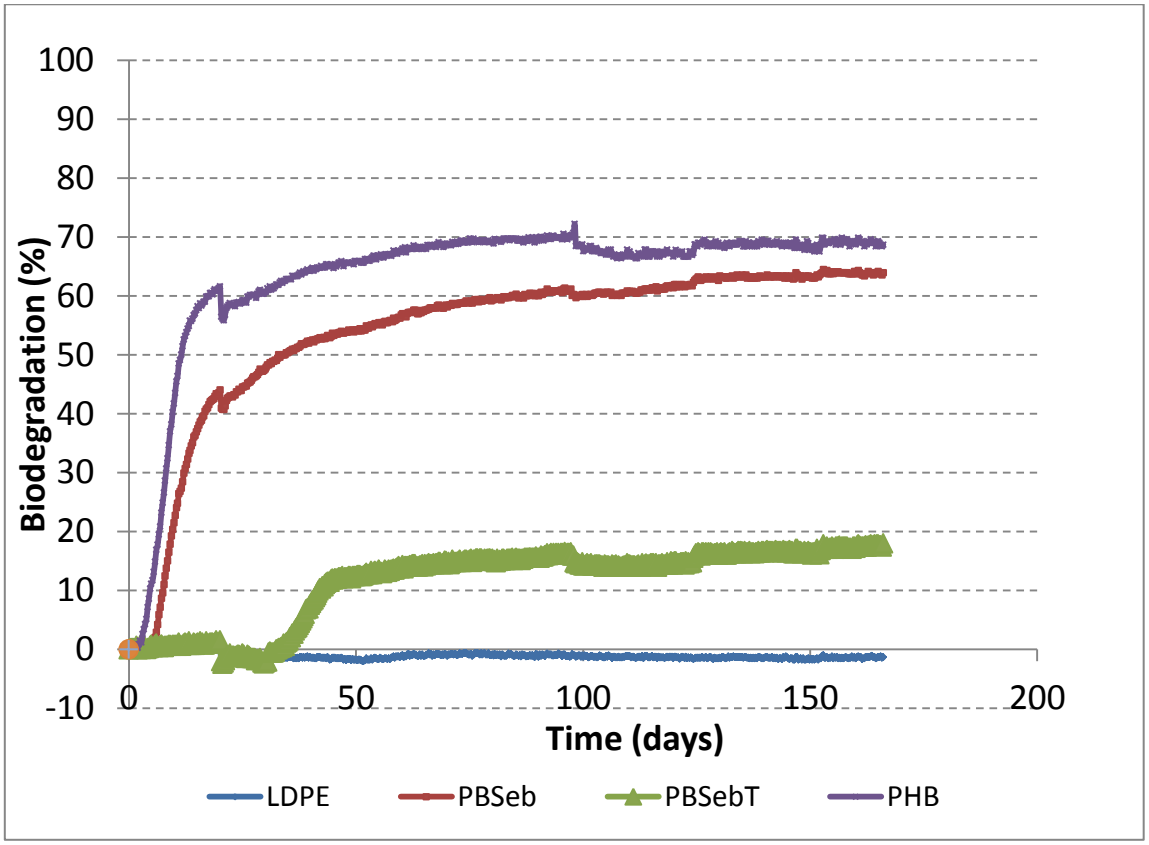


Figure 97. Biodegradation trends in pelagic environments (free water from Salamina island, GK) measured using OXITOP® system (Oxygen depletion)

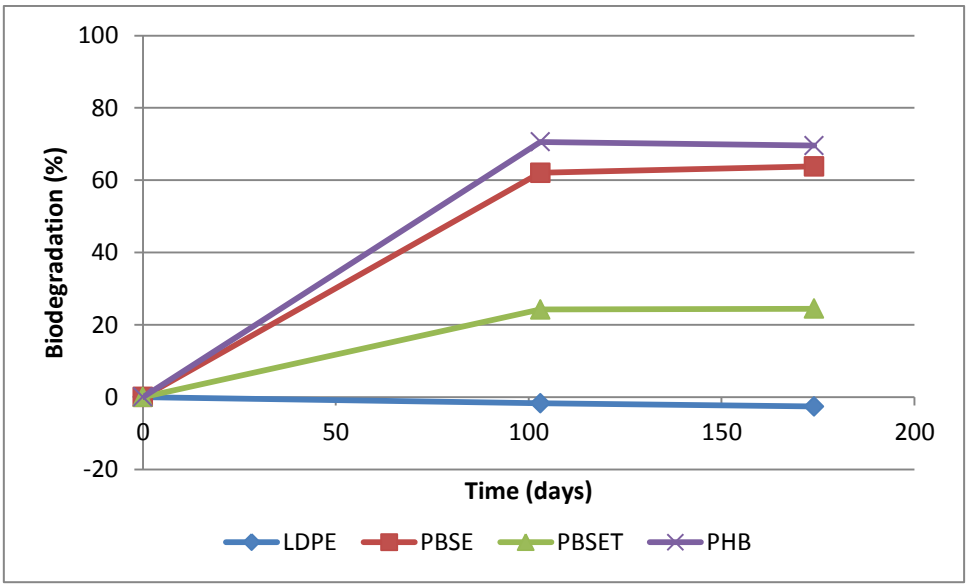


Figure 98. Biodegradation trends in pelagic environments (free water from Salamina island, GK) measured by titration of CO_2 trap of OXITOP® system (Oxygen depletion)

Table 44. O_2 consumption, CO_2 production, biodegradation achieved by each reactor calculated by both systems and biodegradation average plus standard deviation

Test material	O_2 Consumption (mg/l)	Biodegradation (%)	Biodegradation Averages (%)	Biodegradation calculated by titration (%)	Biodegradation averages (by titration)
Control	28.5	-	-	-	-
Control	-2.8	-			
Control	14.2	-			
LDPE	-42.6	-6.72	-1.32 (±7.09)	-7.15	-2.61 (±5.80)
LDPE	-22.7	-3.95		-4.61	
LDPE	73.9	6.70		3.92	
PBSe	349.6	65.30	63.95 (±2.66)	64.72	63.82 (±2.28)
PBSe	327.2	60.89		61.21	
PBSe	352.4	65.68		65.47	
PBSeT	247.0	46.31	17.99 (±24.54)	64.44	24.45 (±35.09)
PBSeT	37.0	4.59		10.11	
PBSeT	28.4	3.08		-1.19	
PHB	238.2	71.46	68.70 (±6.32)	65.06	69.55 (±4.87)
PHB	258.5	73.17		68.88	
PHB	218.3	61.46		74.72	

As reported in Table 44 PBSeT highlighted a high standard deviation and a low level of biodegradation. Figure 99 shows the strange behaviour that characterized the PBSeT test material. In two reactors the biodegradation of PBSeT never started but in one reactor, after a lag phase of around 30 days, a certain biodegradation level was achieved (around 50%). To improve the biodegradation capacity of the two reactors that did not biodegrade, 5 ml of R10 were added to both in order to try to inoculate them with an active microbial consortium able to biodegrade the PBSeT. Unfortunately this experiment did not lead to any appreciable result.

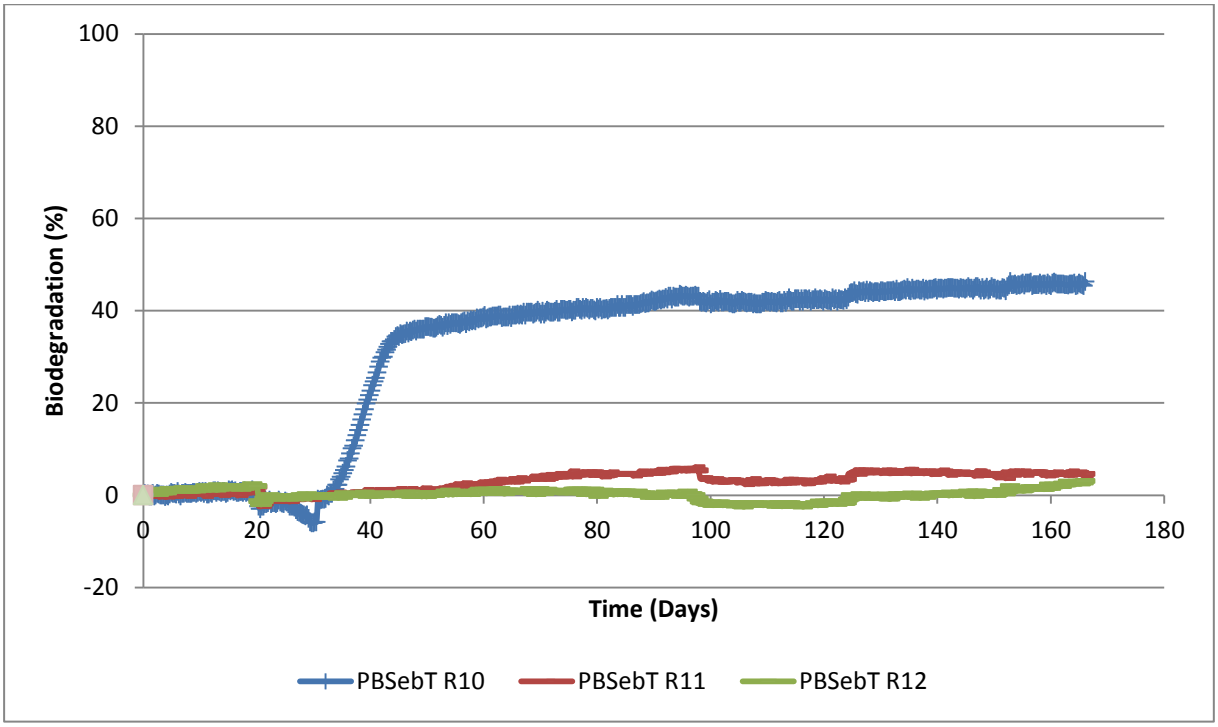


Figure 99. Biodegradation trends in pelagic environments (free water from Salamina island, GK) of PBSeT test material measured by OXITOP® system (Oxygen depletion)

In the same way, the biodegradation test performed using the seawater form Elba island highlighted that the Oxitop system was able to measure the biodegradation of the test items by monitoring the O_2 consumption. Also, in this case LDPE did not show any biodegradation and PHB reached the plateau phase around 55-60 days (see Figure 100). The KOH titration, as double check, confirmed the same results obtained by the Oxygen consumption (Figure 101). Also in this case, the polyester test material (PBSe instead PBSeT), showed a conflicting behaviour (Figure 102). One reactor (R9) developed the microbial condition to obtain a good biodegradation but the other two did not lead this performance. Consequently, the biodegradation of PBSe and PBSeT is characterised by a high standard deviation (Table 45).

Once again (as the first year) the cellulose showed a very limited biodegradation. In ASTM D6691-01 (*Biodegradation of plastic materials in the marine environment – natural seawater*) the validity criteria for reference material is biodegradation >70%. This experiment would be not valid due to low biodegradation of cellulose. On the contrary, PHB test material exceeds widely this limit. This issue remaining an open point. The use of aquarium microbial inoculum, in order to increase the microbial population, could help the activity of natural seawater and as a consequence the total biodegradation of the reference material.

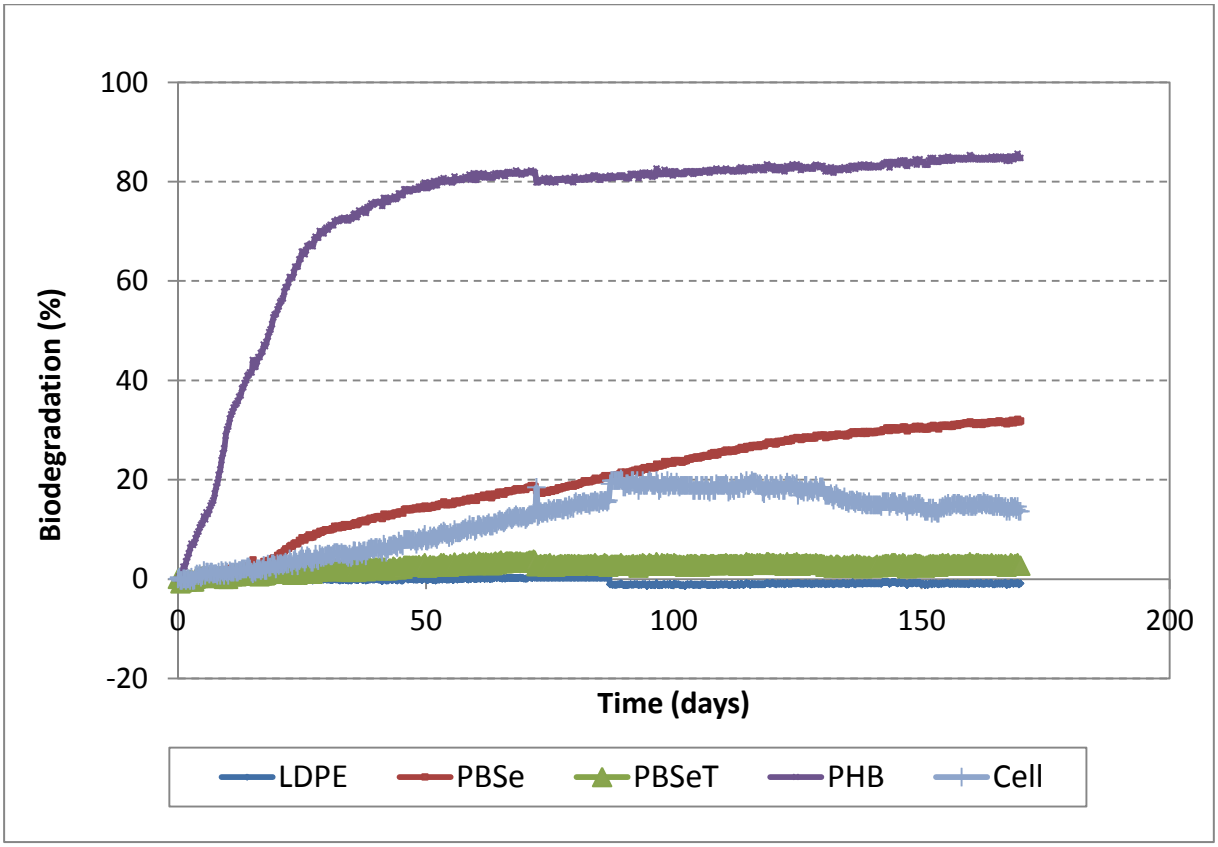


Figure 100. Biodegradation trends in pelagic environments (free water from Elba) measured using OXITOP® system (Oxygen depletion)

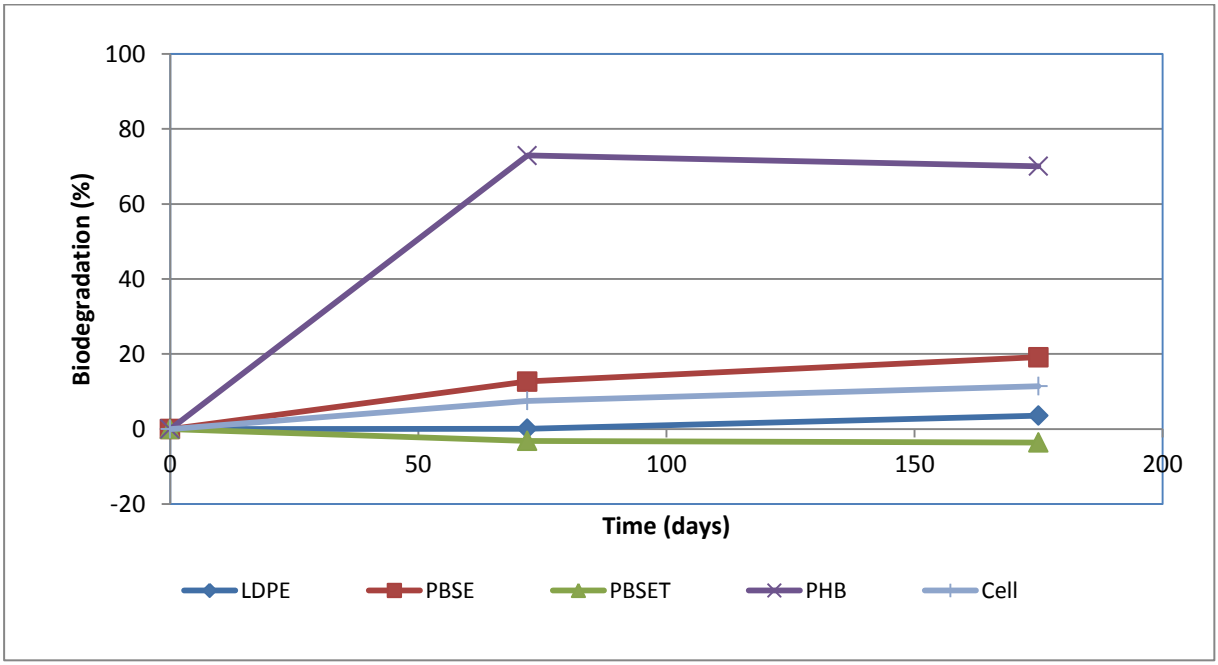


Figure 101. biodegradation trends in pelagic environments (free water from Elba) measured by titration of CO_2 trap of OXITOP® system (Oxygen depletion)

Table 45. O_2 consumption, CO_2 production, biodegradation achieved by each reactor calculated by both systems and biodegradation average plus standard deviation

Test material	O_2 Consumption (mg/l)	Biodegradation (%)	Biodegradation Averages (%)	Biodegradation calculated by titration (%)	Biodegradation averages (by titration)
Control	-19.90	-	-	-	-
Control	-19.90	-			
Control	-22.80	-			
LDPE	-31.30	-1.21	-0.87±0.34	7.54	3.57±4.67
LDPE	-25.60	-0.52		-1.58	
LDPE	-28.40	-0.87		4.73	
PBSe	25.60	8.29	31.82±37.07	-2.25	19.14±32.10
PBSe	51.10	12.63		3.60	
PBSe	372.00	74.55		56.05	
PBSeT	-5.70	2.70	2.65±0.83	-16.04	-3.61±10.94
PBSeT	-2.90	3.49		0.68	
PBSeT	-11.30	1.79		4.53	
PHB	287.50	86.67	84.72±3.12	72.12	70.06±10.84
PHB	252.70	81.14		58.34	
PHB	276.20	86.41		79.72	
Cell	37.00	20.10	13.58±9.87	39.76	11.44±25.02
Cell	31.30	18.41		-7.67	
Cell	-14.20	2.22		2.23	

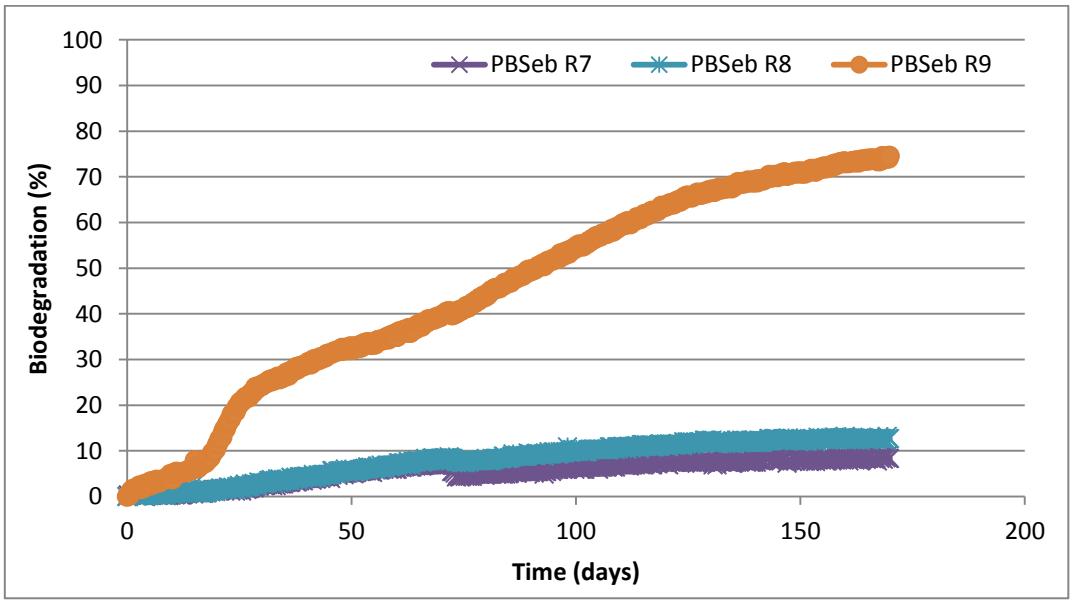


Figure 102. Biodegradation trends in pelagic environments (free water from Elba) of PBSe test material measured by OXITOP® system (Oxygen depletion)

6.2 Laboratory results: OWS

6.2.1 Biodegradation in Eulittoral zone

A sandy sediment has been withdrawn from the eulittoral zone of the shoreline in Greece and Italy by the Agricultural University of Athens and Hydra Institute for Marine Science, respectively (date of receipt Greece: 16/09/2015 - date of receipt Italy: 07/07/2015). The sandy sediments were stored at approximately 4°C before use. The experiments were carried out in the same conditions of the first year with only one modification: nutrient solution was added to the sediment in order to reach a concentration of 0.1 mg N per mg test material organic carbon for the Italian sediment, while for the Greek sediment an average TOC of 50% was assumed for all samples and the same amount of nutrient solution was added to all reactors. The fact that a different approach was used for both sediments was caused by the addition of a new note in the draft standard test method. Solutions of 40 mL KOH (0.05 N) and 0.05 N HCl were used to execute the titrations.

Remark: Due to the limited amount of received sediment, only 300 g of Greek sediment was added per reactor. The test item amount was not changed as in the test methodology a test item amount is given (100 mg) and no test item concentration.

Characterization of sediments (2015-2016)

The results of the chemical analyses executed on the marine sediments are given in Table 46. The analyses on the Italian sediment were executed on the sediment without addition of nutrient solution. The volatile solids content of the sediment of Greece (2.9%) was considerably higher when compared to the sediment of Italy (0.6%). As mentioned before, the sediment were also characterized by ISA group and data are reported in Table 37.

Table 46. Characteristics of the sediments

Characteristics	Greece	Italy
Dry matter (DM, %)	86.0	78.5
Moisture content (%)	14.0	21.5
Volatile solids (VS, % on DM)	2.9	0.6
Ash content (% on DM)	97.1	99.4
pH	8.6 (no nutrient solution) 8.8 (with nutrient solution)	8.5
EC (µS/cm)	3860 (no nutrient solution) 3550 (with nutrient solution)	2820
Total N (g/kg DM)	0.6	0
NH_4^+-N (mg/L)	< 9 (with and without nutrient solution)	< 9
NO_X^--N (mg/L)	< 5 (no nutrient solution) 29 (with nutrient solution)	< 5 (no nutrient solution) 18 (0.725 ml nutrient solution)

		32 (1.3 ml nutrient solution)
P (g/kg DM)	0.1	7.2
K (g/kg DM)	0.5	1.4
Mg (g/kg DM)	12.7	2.5
Ca (g/kg DM)	261.7	3.6
C/N ratio	24	Not possible to calculate

Sandy marine sediment from Greece: Biodegradation results

Figure 103 shows the evolution of the biodegradation of the different samples in sandy marine sediment from Greece. The test was stopped after 240 days for all reactors. No biodegradation was observed for LDPE. A rather comparable biodegradation was observed for PHB copolymer and PBSe, while the biodegradation of PBSeT was somewhat lower. At the end of the test the biodegradation percentages of the samples were -4.9% ± 1.4% for LDPE, 59.0% ± 4.9% for PHB copolymer, 59.4% ± 10.0% for PBSe and 39.1% ± 2.0% for PBSeT. The standard deviations in this test are acceptable. When comparing the results of the second run (= with additional of nutrients) with the first run (= without nutrients), it is noted that biodegradation of PBSe and PBSeT is proceeding faster in the second run when compared to the first run. This might be caused by the addition of the nutrients.

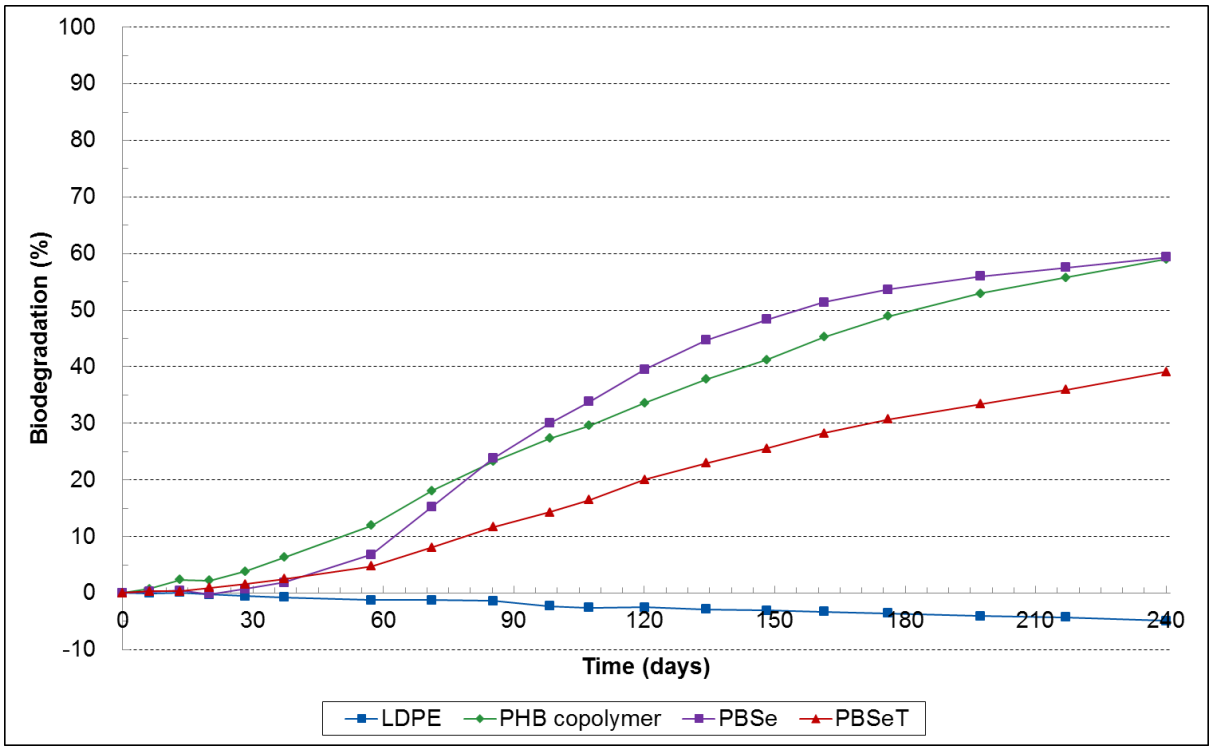


Figure 103. Evolution of biodegradation in sandy marine sediment (Greece)

The evolution of the net cumulative CO_2 production of the individual replicates is given in Figure 104 up to Figure 108. At the end of the test (= after 240 days), the blank

reactors have reached a net cumulative CO_2 production of 206 mg ± 8 mg. The net cumulative CO_2 production of the samples were 191 mg ± 8 mg for LDPE, 310 mg ± 12 mg for PBH copolymer, 357 mg ± 40 mg for PBSe and 312 mg ± 13 mg for PBSeT.

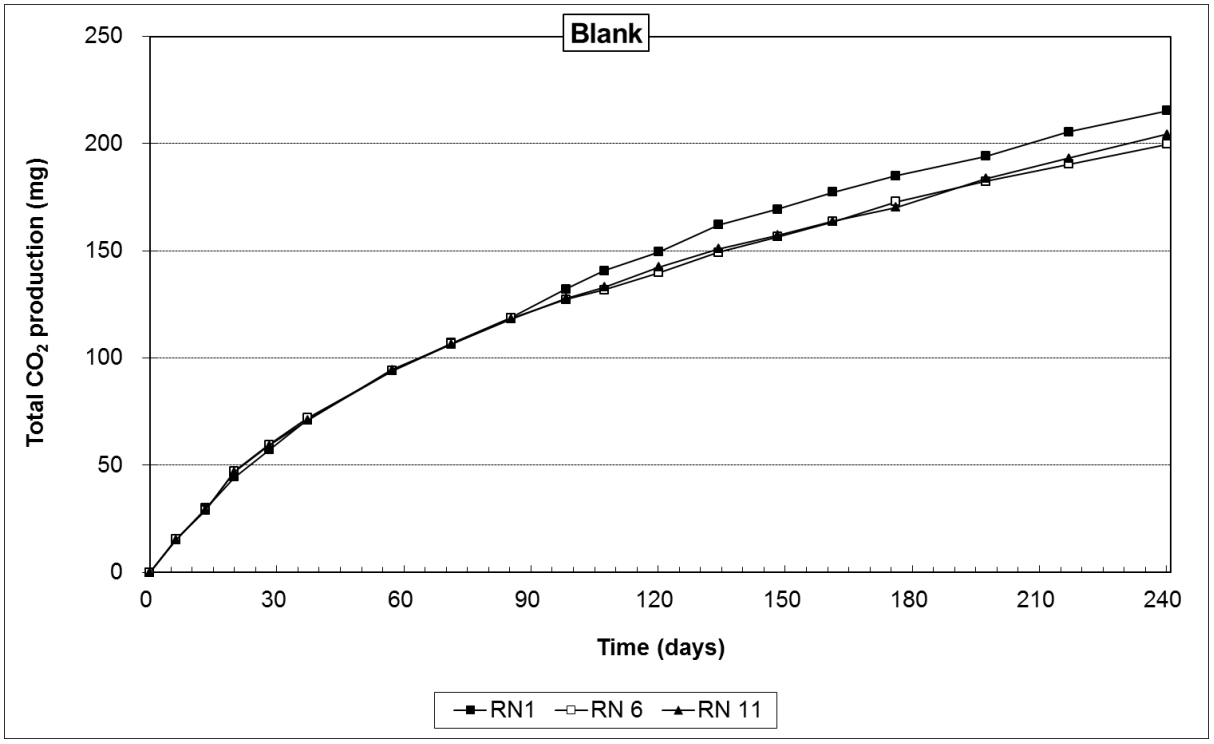


Figure 104. Total CO_2 production in blank reactors in sandy marine sediment (Greece)

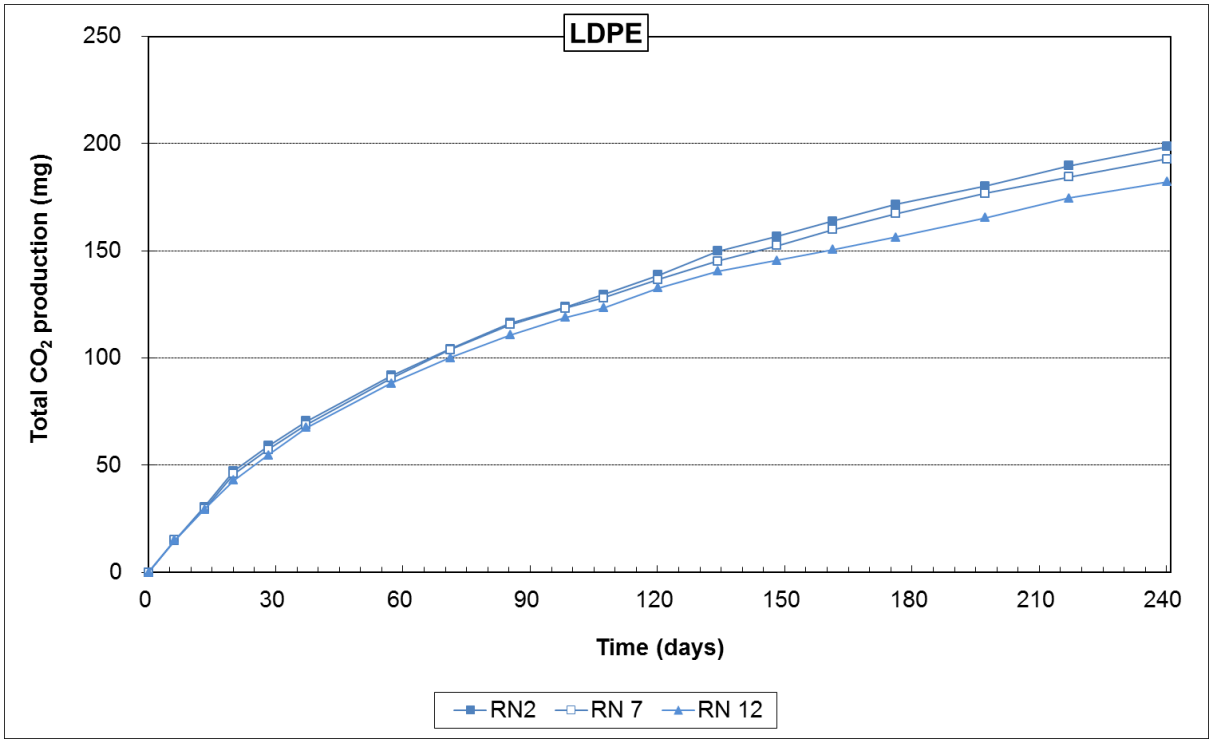


Figure 105. Total CO_2 production in LDPE reactors in sandy marine sediment (Greece)

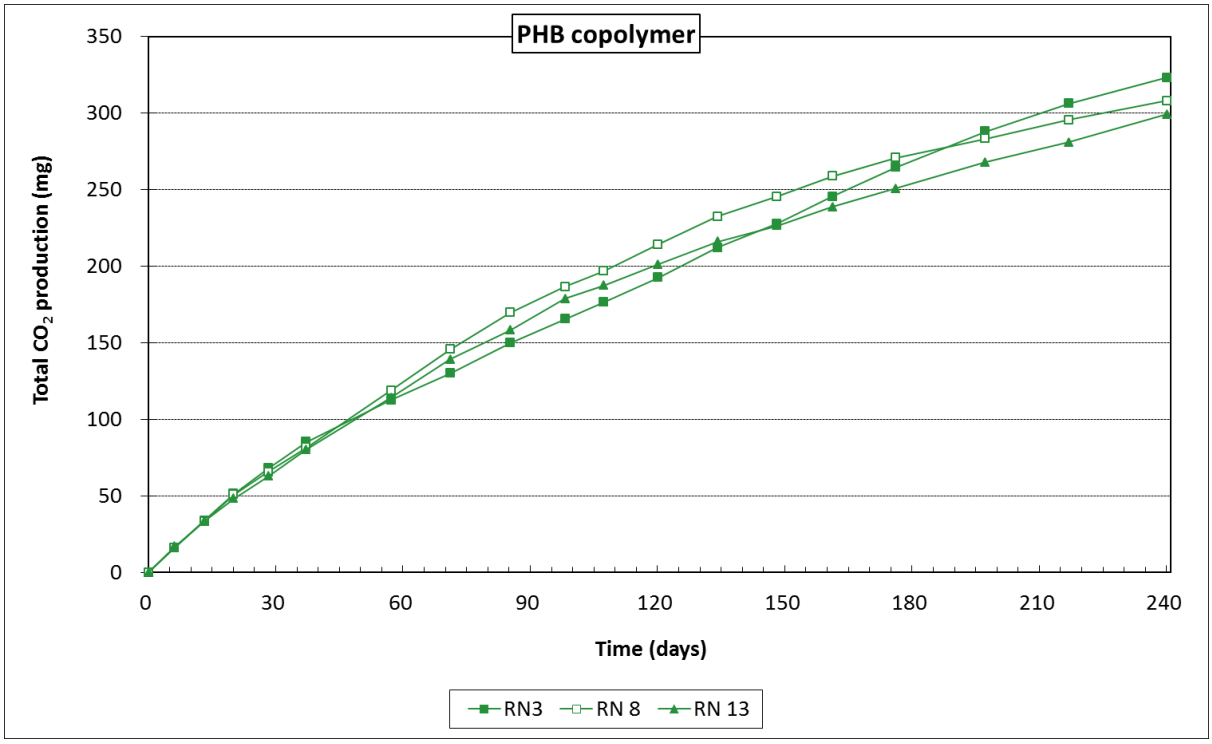


Figure 106. Total CO_2 production in PHB copolymer reactors in sandy marine sediment (Greece)

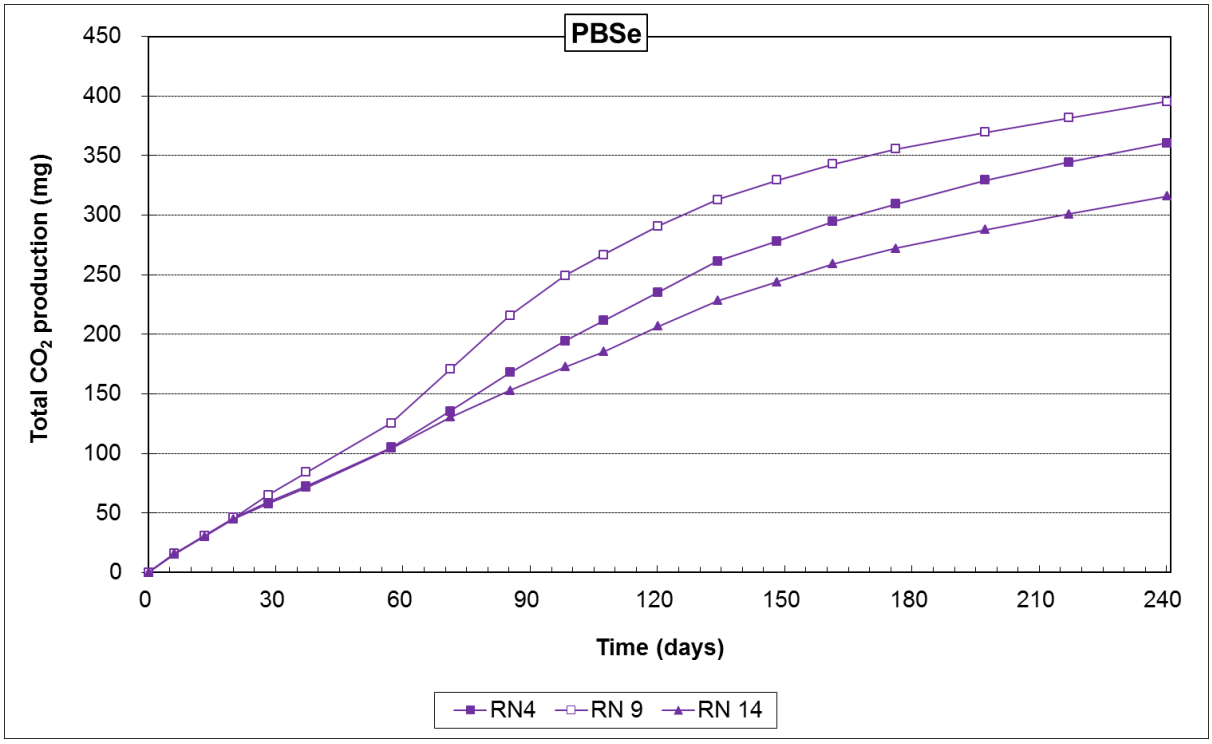


Figure 107. Total CO_2 production in PBSe reactors in sandy marine sediment (Greece)

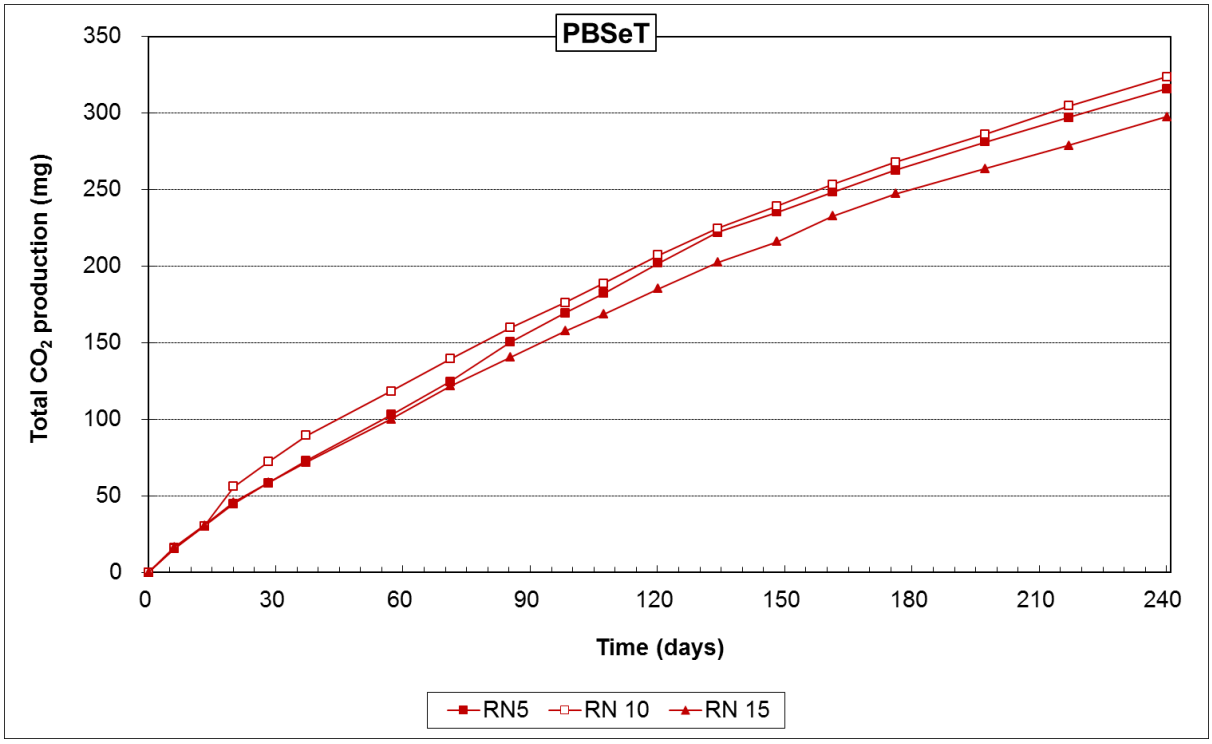


Figure 108. Total CO_2 production in PBSeT reactors in sandy marine sediment (Greece)

At the end of the test (= after 240 days) the remaining test material was manually retrieved, dried and weighted. An overview of the biodegradation percentages and the

disintegration percentages is given in Table 47 and pictures are shown in Figure 109. The disintegration percentages are clearly higher when compared to the disintegration percentages. The disintegration percentages for PBSe and PBSeT are much higher in the second run when compared to the first run. This can be caused by the nutrient addition.

Table 47. Description and disintegration percentage of retrieved test materials after 240 days

Test item	Biodegradation	Disintegration
LDPE	4.9% ± 1.4% RN2 = -2.5% // RN7 = -4.5% // RN12 = -7.7%	0.2% ± 0.4% RN2 = -0.3% // RN7 = 0.5% // RN12 = 0.3%
PHB copolymer	59.0% ± 4.9% RN3 = 64.9% // RN8 = 58.0% // RN13 = 54.1%	87.5% ± 9.8% RN3 = 91.3% // RN8 = 94.8% // RN13 = 76.3%
PBSe	59.4% ± 10.0% RN4 = 60.2% // RN9 = 74.4% // RN14 = 43.6%	94.5% ± 6.4% RN4 = 96.0% // RN9 = 100.0% // RN14 = 87.5%
PBSeT	39.1% ± 2.0% RN5 = 40.2% // RN10 = 43.0% // RN15 = 34.3%	96.4% ± 3.2% RN5 = 95.3% // RN10 = 93.9% // RN15 = 100.0%

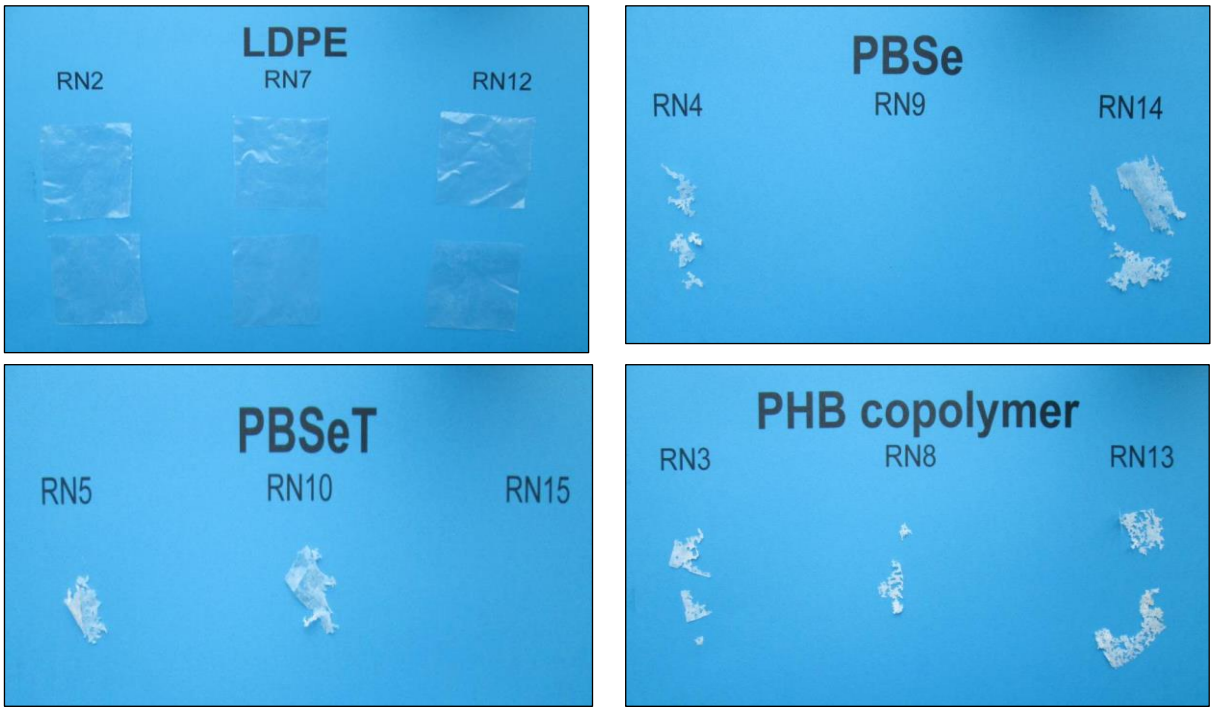


Figure 109. Overview of retrieved pieces of LDPE, PBSe, PBSeT and PHB

At the end of the test some chemical analyses were performed on the content of the reactors (Table 48). It is noted that the volatile solids (VS) content had decreased during the

test. At start a value of 2.9% on dry weight basis was measured, while the VS content at the end of the test (= after 240 days of incubation) varied between 1.1% and 2.7%. Moreover, it is observed that the pH had increased from a value of 8.8 at start of the test till values varying between 9.0 and 9.2. The nitrate content is some reactors was higher when compared to the measured nitrate content at start of the test (= 29 mg/l).

Table 48. Results chemical analyses performed at the end of the test (240 days) (DM = Dry matter; VS = Volatile solids)

	DM (%)	VS (%)	Total N* (mg/kg wet weight)	pH	EC (µS/cm)	NH_4^+-N (mg/l)	NO_X^--N (mg/l)
RN 1 - blank	86.1	1.7	277	9.1	2760	< 10	32
RN 6 - blank	84.3	1.1	90	9.2	2740	< 10	39
RN 11 - blank	84.2	2.3	399	9.2	2900	< 10	30
RN 2 - LDPE	83.5	1.9	100	9.0	2890	< 10	34
RN 7 - LDPE	85.1	1.9	54	9.1	3230	< 10	47
RN 12 - LDPE	83.0	1.3	686	9.1	3160	< 10	38
RN 3 - PHB	83.5	1.8	204	9.2	3270	< 10	46
RN 8 - PHB	84.9	1.8	315	9.0	3100	< 10	28
RN 13 - PHB	84.6	1.9	381	9.2	3210	< 10	45
RN 4 - PBSe	82.6	1.7	206	9.2	2980	< 10	36
RN 9 - PBSe	83.1	2.1	63	9.2	3190	< 10	23
RN 14 - PBSe	84.4	2.0	4	9.2	3310	< 10	44
RN 5 - PBSeT	82.6	1.2	434	9.1	3390	< 10	34
RN 10 - PBSeT	83.2	2.7	320	9.2	3220	< 10	37
RN 15 - PBSeT	80.2	2.3	57	9.2	3320	< 10	11

*Informative values. Normally values below 2300 mg/kg are not reported.

Sandy marine sediment from Italy: Biodegradation results

Figure 110 shows the evolution of the average biodegradation of the different samples in sandy marine sediment from Italy. The test was stopped after 271 days for PHB copolymer, while the test was stopped after 313 days for the other reactors. The difference in the average biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. Negligible biodegradation was observed for LDPE. After 271 days PHB copolymer has reached a biodegradation percentage of 85.5% ± 61.0%. The PHB series was characterized by a very high standard deviation. The biodegradation percentages of the individual replicates were 149.7%, 78.3% and 28.3%. The first replicate can clearly be considered as an unrealistic value (> 100% biodegradation). The average biodegradation pattern without this unrealistic

value is given in Figure 111. Without this outlier, an average biodegradation percentage of 53.3% ± 35.3% is reached after 217 days for PHB copolymer. It was noted that the sediment of the replicate with the lowest biodegradation was characterized a higher humidity when compared to the other replicates. Possibly the lower humidity has delayed the biodegradation. The biodegradation percentages of the other samples at the end of the test (= after 313 days) were 4.3% ± 4.1% for LDPE, 56.1% ± 26.2% for PBSe and 53.9% ± 11.8% for PBSeT.

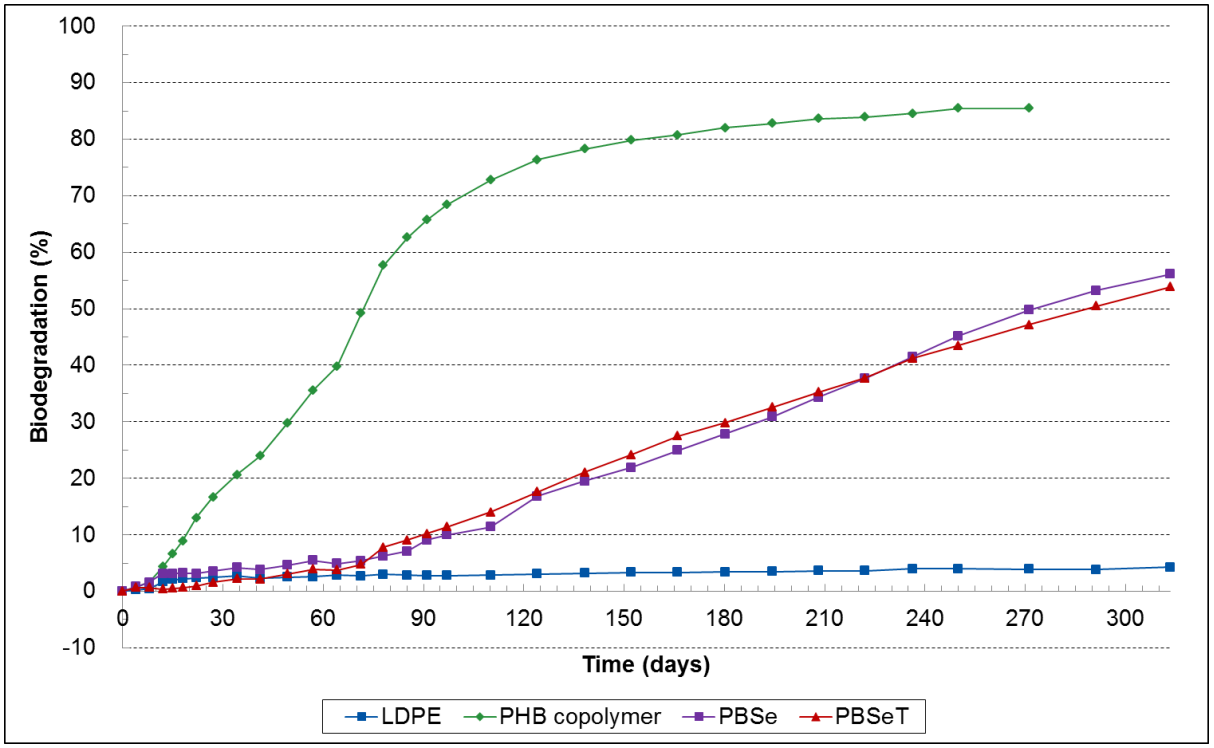


Figure 110. Evolution of biodegradation in sandy marine sediment (Italy)

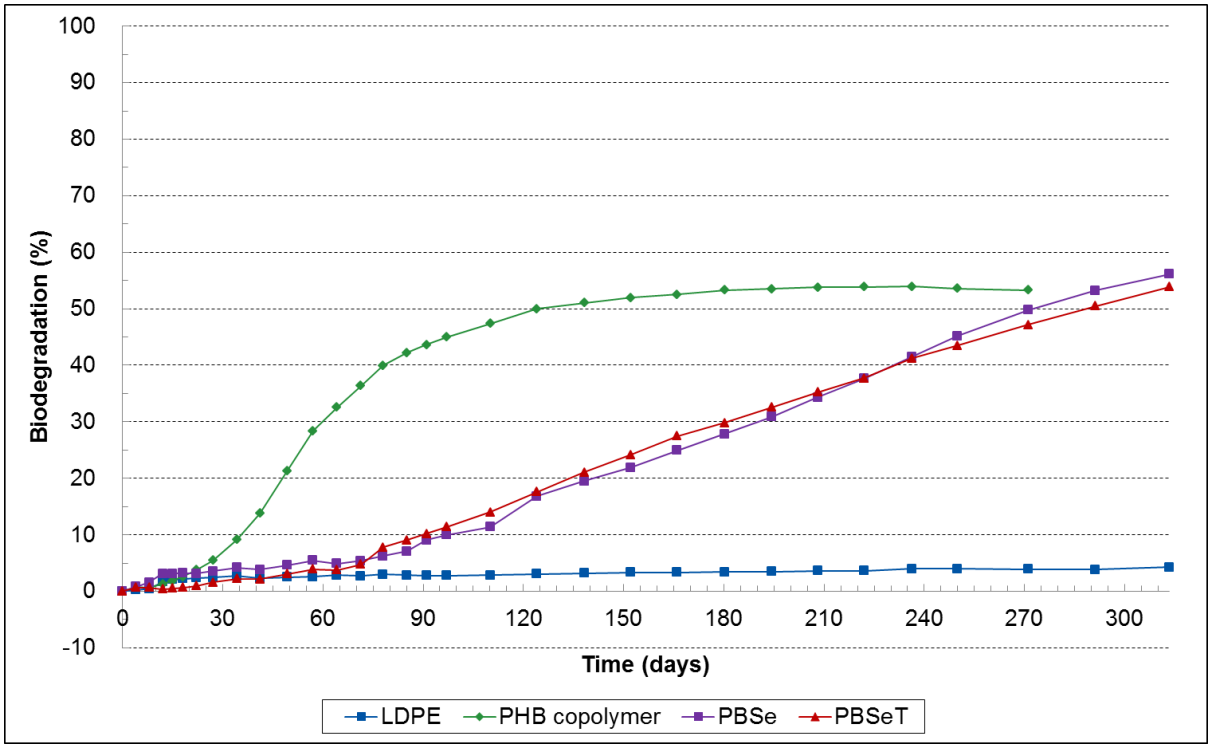


Figure 111. Evolution of biodegradation in sandy marine sediment (Italy) (without unrealistic value for one PHB replicate)

The evolution of the net cumulative CO_2 production of the individual replicates is given in Figure 112 up to Figure 116. At the end of the test (= after 271 days for PHB copolymer and after 313 days for the other series), the blank reactors have reached a net cumulative CO_2 production of 183 mg ± 23 mg. The net cumulative CO_2 production of the samples were 195 mg ± 11 mg for LDPE, 319 mg ± 101 mg for PHB copolymer, 311 mg ± 58 mg for PBSe and 313 mg ± 26 mg for PBSeT.

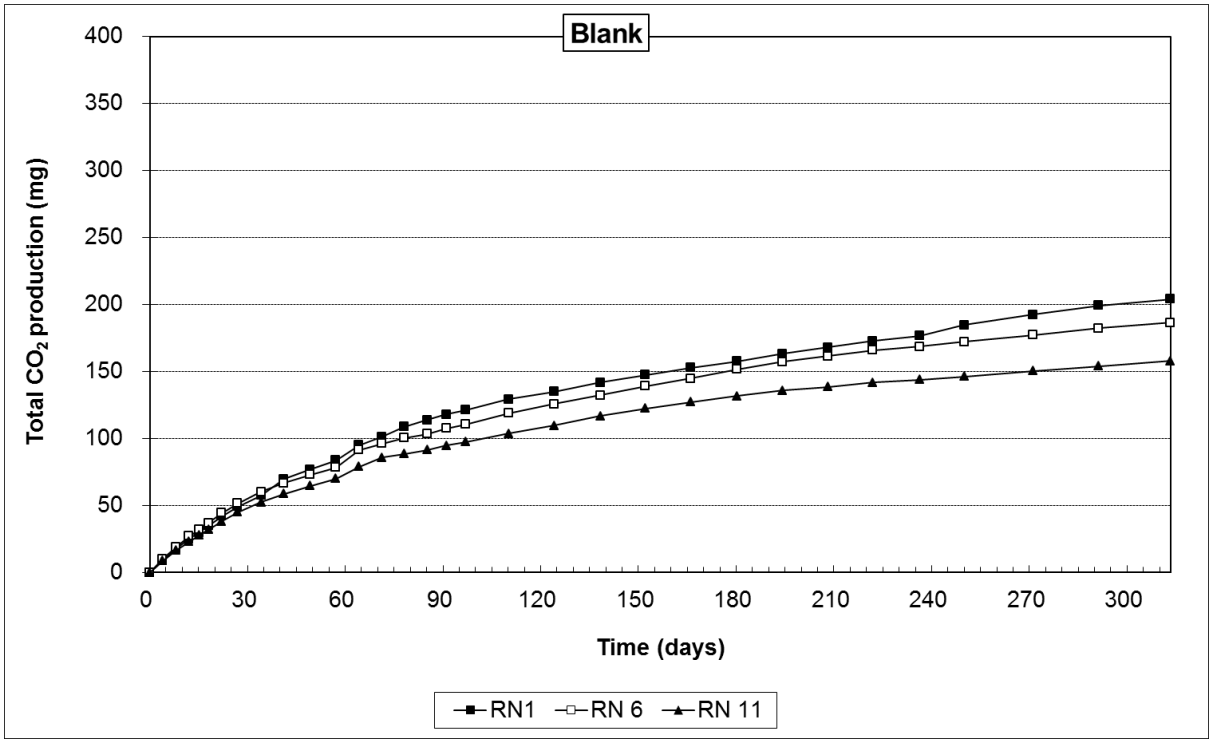


Figure 112. Total CO_2 production in blank reactors in sandy marine sediment (Italy)

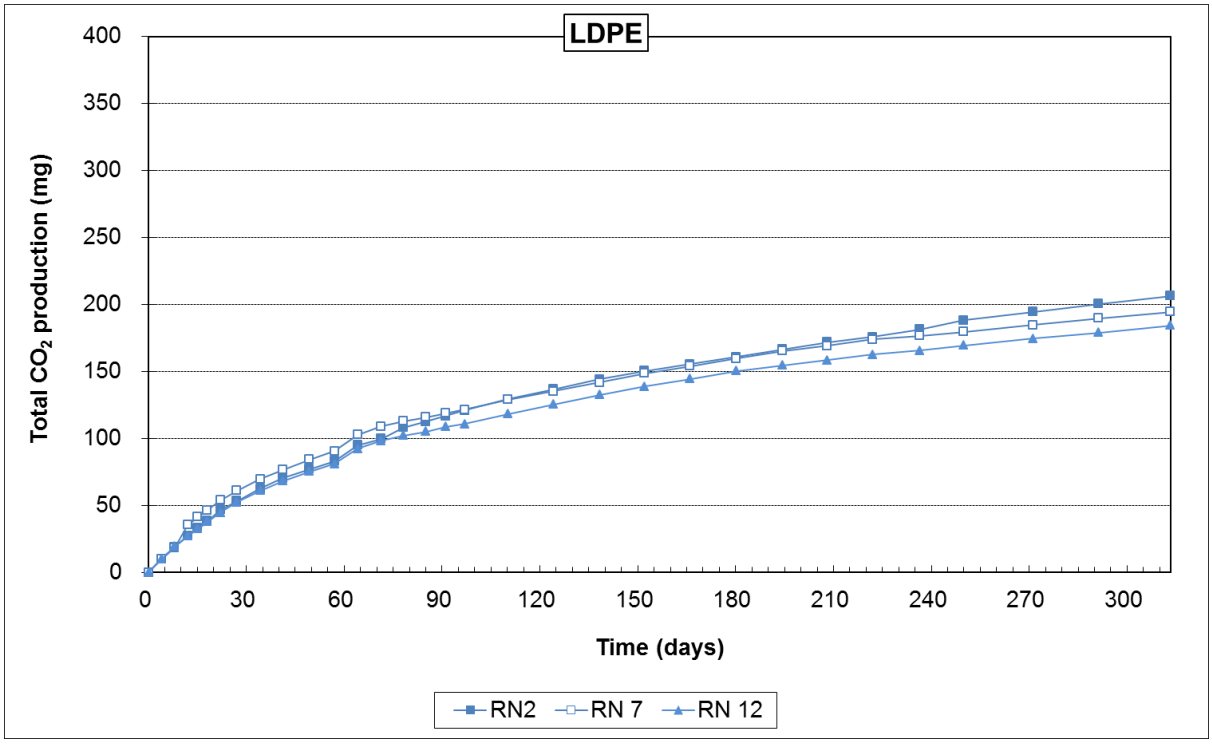


Figure 113. Total CO_2 production in LDPE reactors in sandy marine sediment (Italy)

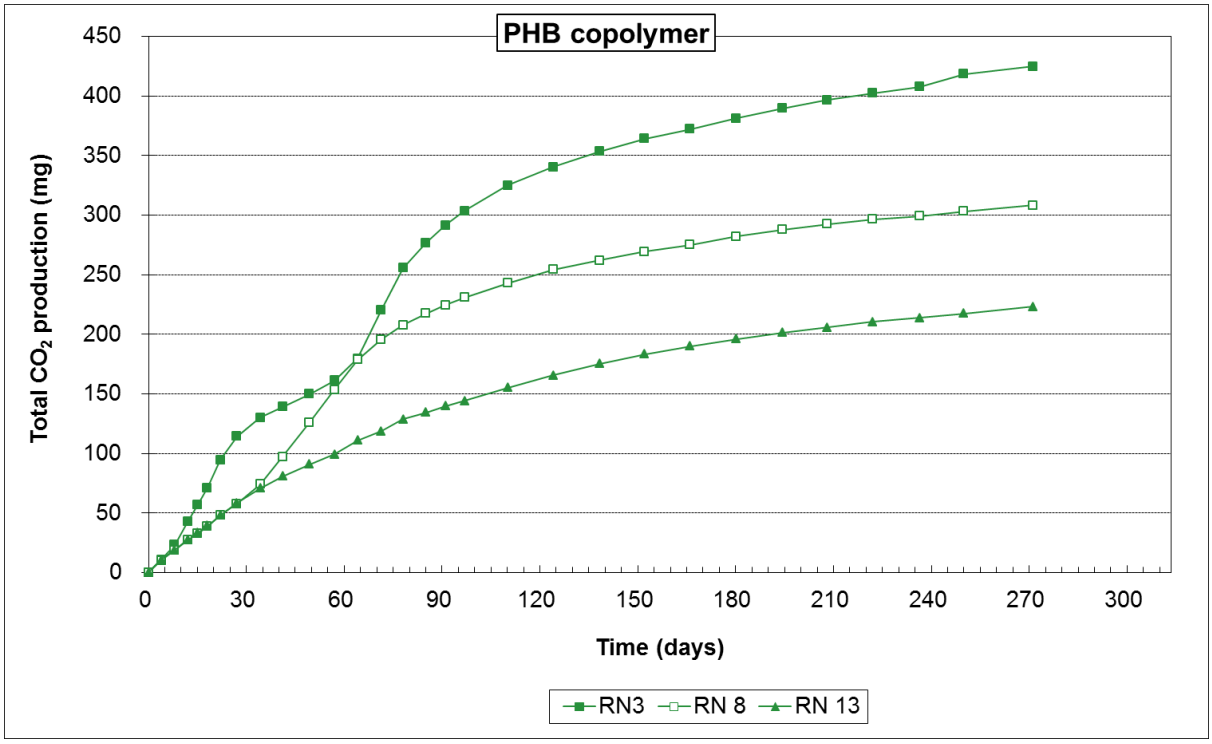


Figure 114. Total CO_2 production in PHB copolymer reactors in sandy marine sediment (Italy)

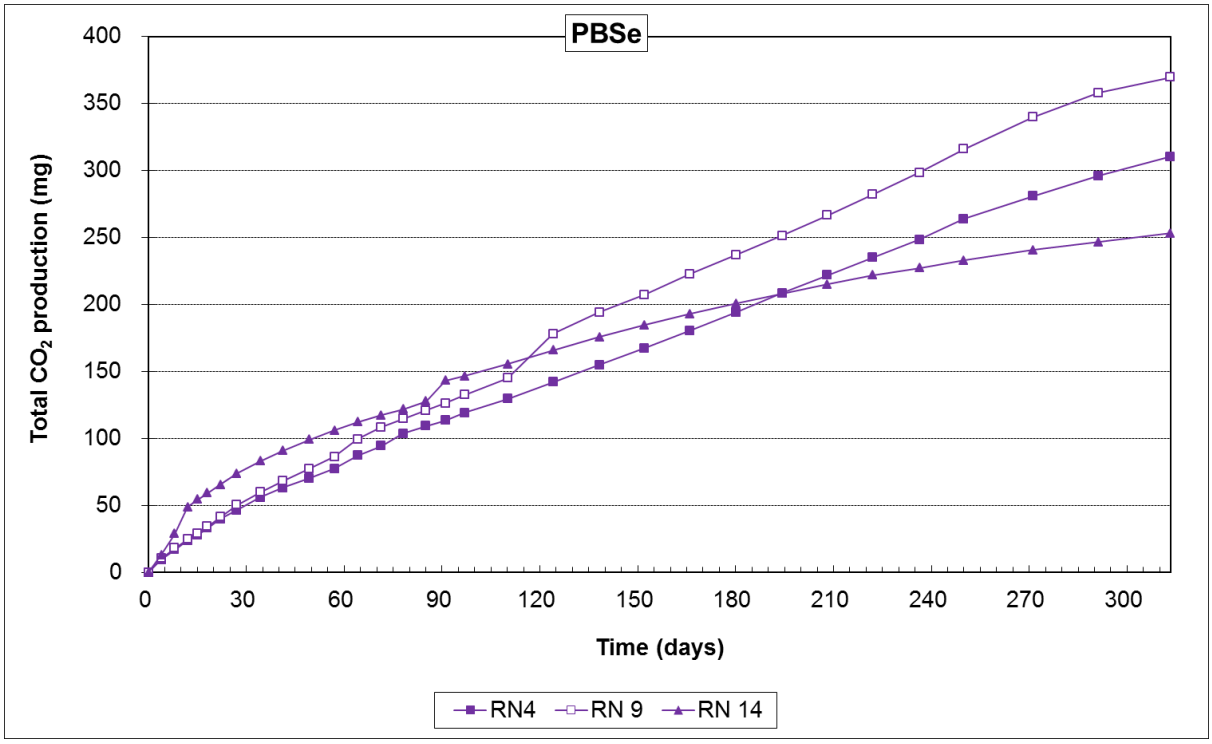


Figure 115. Total CO_2 production in PBSe reactors in sandy marine sediment (Italy)

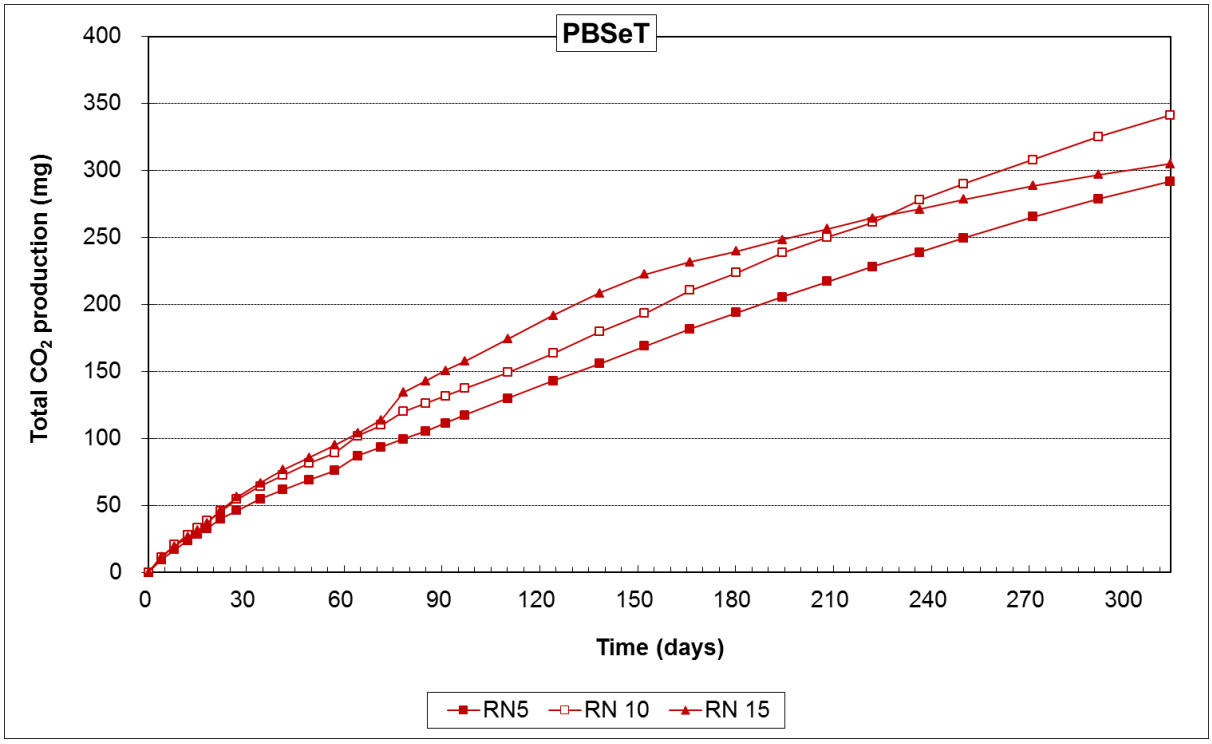


Figure 116. Total CO_2 production in PBSeT reactors in sandy marine sediment (Italy)

At the end of the test (= after 271 days for PHB copolymer and after 313 days for the other test materials) the remaining test material was manually retrieved, dried and weighted. An overview of the biodegradation and the disintegration percentages is given in Table 49 and pictures are shown in Figure 117. The disintegration percentages of the bioplastics are generally somewhat higher when compared to the biodegradation percentages (especially for PBSe and PBSeT). For PHB copolymer only of 1 replicate sample was retrieved. This was the replicate characterized by the lowest biodegradation.

Table 49. Biodegradation and disintegration percentages of test materials after 271 days (PHB copolymer) and after 313 days

Test item	Biodegradation	Disintegration
LDPE	4.3% ± 4.1% RN2 = 8.6% // RN7 = 3.9% // RN12 = 0.4%	0.7% ± 0.4% RN2 = 0.3% // RN7 = 0.7% // RN12 = 1.1%
PHB copolymer	85.5% ± 61.0% RN3 = 149.7% // RN8 = 78.3% // RN13 = 28.3%	85.9% ± 24.5% RN3 = 100.0% // RN8 = 100.0% // RN13 = 57.6%
PBSe	56.1% ± 26.2% RN4 = 56.1% // RN9 = 82.4% // RN14 = 30.0%	87.0% ± 12.6% RN4 = 74.9% // RN9 = 100.0% // RN14 = 86.0%
PBSeT	53.9% ± 11.8% RN5 = 45.0% // RN10 = 67.3% // RN15 = 49.4%	84.6% ± 19.0% RN5 = 63.4% // RN10 = 90.5% // RN15 = 100.0%

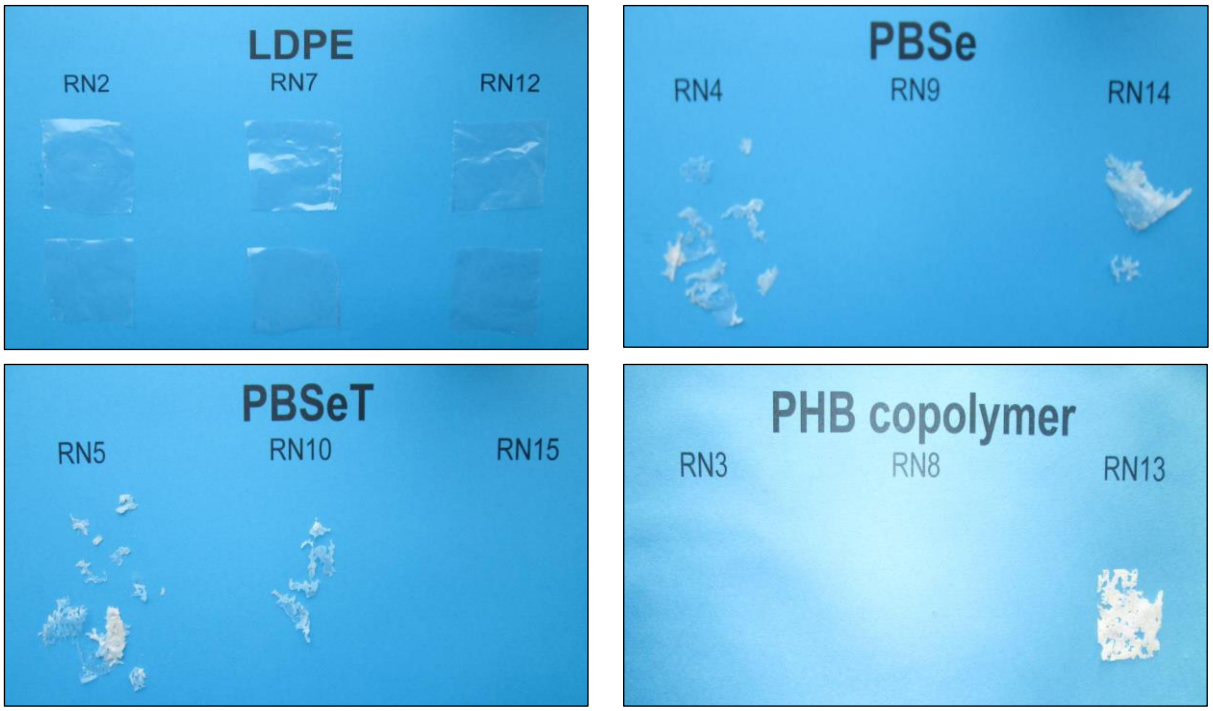


Figure 117. Overview of retrieved pieces of LDPE, PBSe, PBSeT and PHB

At the end of the test some chemical analyses were performed on the content of the reactors (Table 50). The content of the reactors with PHB copolymer were not analysed. The dry matter content varied significantly between the different replicate (range: 75.0% - 83.0%). No large variations were observed between the volatile solids content of the different replicates. The electrical conductivity (EC) varied significantly between the replicates (range: 2800 µS/cm - 8300 µS/cm). When compared to the start of the test (VS = 0.6%), the VS content remained identical or had slightly decreased. Also the pH remained rather stable during the test.

Table 50. Results chemical analyses performed at the end of the test (313 days) (DM = Dry matter; VS = Volatile solids)

	DM (%)	VS (%)	Total N* (mg/kg wet weight)	pH	EC (µS/cm)	NH_4-N (mg/l)	NO_X-N (mg/l)
RN 1 - blank	82.3	0.5	550	8.6	2800	< 10	30
RN 6 - blank	83.0	0.5	207	8.7	2930	< 10	32
RN 11 - blank	77.9	0.6	33	8.7	4400	< 10	36
RN 2 - LDPE	80.9	0.5	280	8.6	3570	< 10	42
RN 7 - LDPE	79.5	0.5	462	8.5	3740	< 10	65
RN 12 - LDPE	76.9	0.6	203	8.7	4380	< 10	47

RN 4 - PBSe	76.1	0.6	144	8.8	4640	< 10	48
RN 9 - PBSe	77.4	0.6	75	8.7	5080	< 10	40
RN 14 - PBSe	78.4	0.6	794	8.7	4550	< 10	< 10
RN 5 - PBSeT	78.5	0.5	576	8.7	8300	< 10	36
RN 10 - PBSeT	78.2	0.6	359	8.6	4740	< 10	37
RN 15 - PBSeT	75.0	0.6	824	8.6	5300	< 10	10

*Informative values. Normally values below 2300 mg/kg are not reported.

Conclusions and recommendations

A summary of the biodegradation and disintegration percentages is given in Figure 118 and Figure 119, respectively.

Main observations:

- No or negligible biodegradation is measured for the negative reference material LDPE. This indicates that the test method is reliable.
- Biodegradation of PHB copolymer is generally higher when compared to PBSe, while biodegradation of PBSe is generally higher when compared to PBSeT (as expected).
- The addition of nutrients (= year 1 (without nutrients) versus year 2 (with nutrients)) increases the biodegradation rate. This is especially observed for PBSe and PBSeT.
- The addition of nutrients also increases the variability between the different replicates. No unrealistic values (e.g. biodegradation percentages > 100%) were observed in year 1 (= without nutrients), while in year 2 an unrealistic value was observed for PHB in the Italian sediment.
- Disintegration percentage of the sample is in general higher when compared to the biodegradation percentage.
- Disintegration of the remaining film pieces can easily be determined. The contamination with sand is rather low or negligible. Only for PBSeT in the Italian sediment of year 1 a high standard deviation was observed (most probably caused by the fact that due to a human mistake the sample was not retrieved).
- Difference between results in Italian and Greek sediment is rather limited. Only for PHB copolymer a large difference was observed in year 1. The higher biodegradation in the Italian sediment might be caused by the higher nitrogen content and the lower C/N ratio in the Italian sediment.

Recommendations:

- Sample is currently added under the form of film. The variability between the replicates can probably be decreased when the sample would be added under the form of **powder**. The disadvantage of this approach is that the disintegration could not be evaluated anymore at the end of the test.

- Sample is currently added in a very low quantity (100 mg per 400 g sediment). It might be better to **increase the test item concentration**. In this way the difference between carbon dioxide production in the blank and test reactors will become larger.
- The **addition of nutrients** will normally increase the biodegradation rate and decrease the duration of the test.
- Include besides a negative reference material also **cellulose as positive reference material** in the test methodology (although it would be advisable to study also the behaviour of cellulose in this system, before including it).

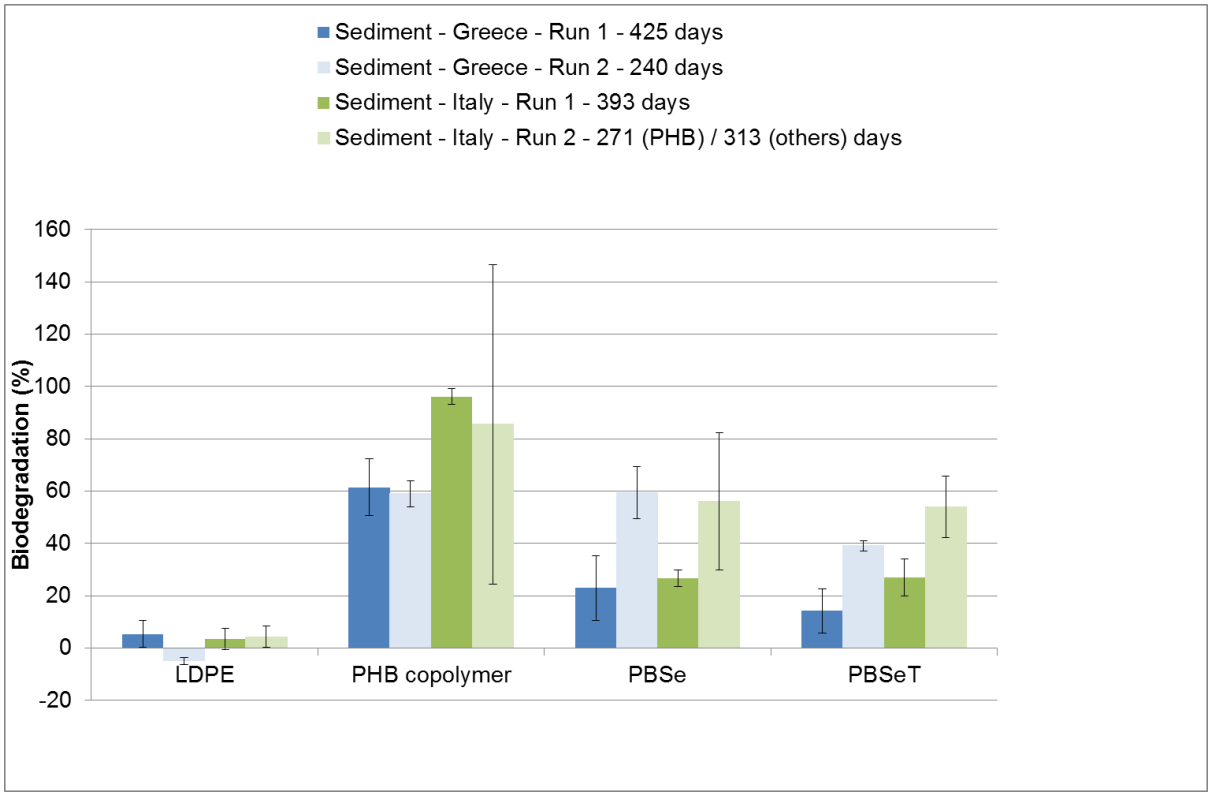


Figure 118. Overview biodegradation percentages at the end of the test

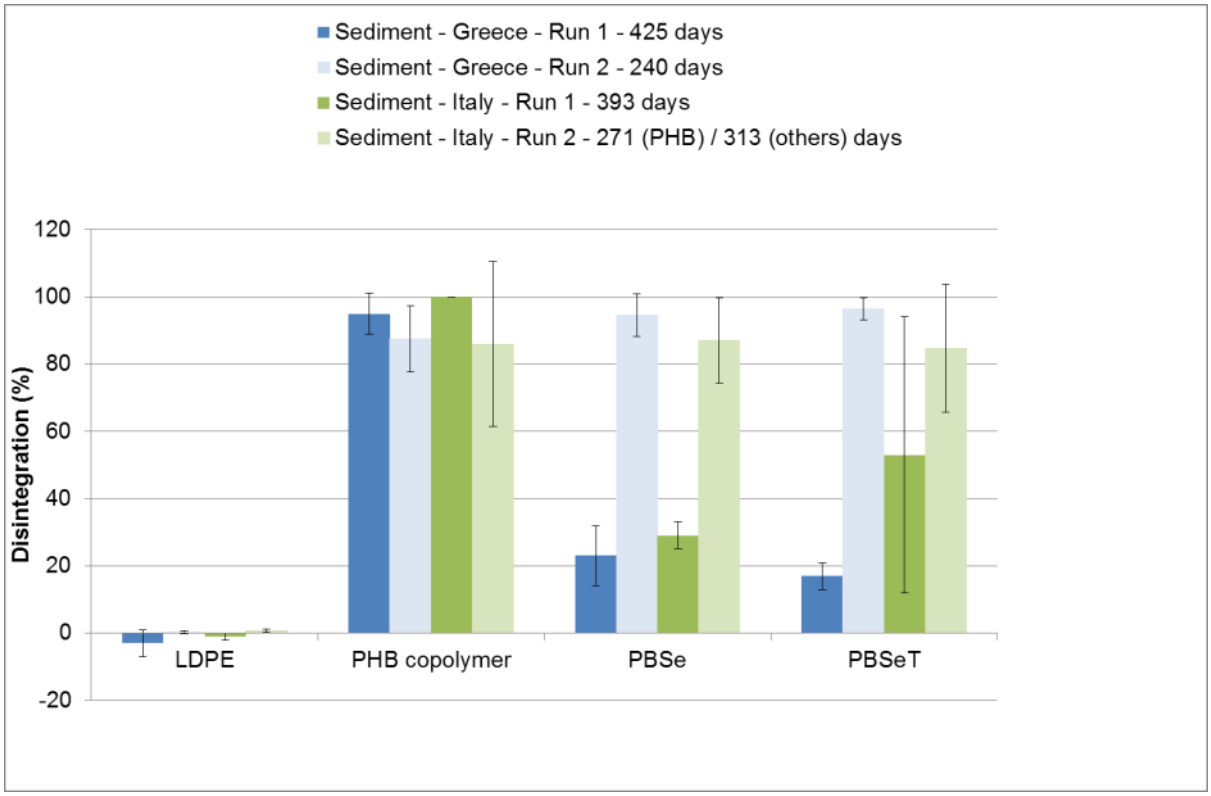


Figure 119. Overview disintegration percentages at the end of the test

6.2.2 Biodegradation in Benthic zone

Seawater/sediment interface – Method by analysis of evolved carbon dioxide

Test set-up

Seawater and a sandy sediment (date of receipt respectively: 16/09/2015 Greek sediment and 07/07/2015 Italian sediment) have been sampled , treated and stored as did in the first year experiment. For the preliminary phase (Greece: started at 30/09/2015 – Italy: started at 12/08/2015), 30 g sediment (15 g fresh sediment and 15 g sediment after calcination) and 70 mL seawater have been measured and put in the test flasks. An homogenous interphase was obtained at the bottom of a reactor between the sediment and the seawater. Subsequently, 1 mL KOH (3 N) was added into the provided compartment of the test flask. These reactors were incubated at a temperature 28°C. The duration of the preliminary phase was 1 week. Similar values were titrated at the end of the preliminary phase. In total, 15 test flasks (each with a 300 mL volume) were prepared. Three flasks for each specimen and 3 control flasks. All samples were cut into circles with varying areas in order to reach a weight of approximately 20 mg. The test materials were placed at the top of the sediment. A mosquito net (circular shape) was added to prevent floating of the test materials.

The period of time between the carbon dioxide analysis by means of titrations were variable. With regard to the KOH solution: 1 mL of KOH solution with a molarity of 3 N was used. The titration was performed with 0.05 N HCl.

Characterization of Open-Bio sediments/seawater

The characteristics of the sediment (= 50% fresh sediment + 50% sediment after calcination) and the final medium (= 70 g seawater + 15 g fresh sediment + 15 g sediment after calcination) are shown in Table 51.

Table 51. Characteristics of sediment and final medium

Characteristics	Sample	Greece	Italy
Dry matter (DM, %)	Sediment	72.1	79.8
Moisture content (%)	Sediment	27.9	20.2
Volatile solids (VS, % on DM)	Sediment	4.2	3.0
Ash content (% on DM)	Sediment	95.8	97.0
pH	Final medium	8.8	9.3
NH_4^+-N (mg/L)	Final medium	< 9	26*
NO_x^--N (mg/L)	Final medium	< 5	< 5
Total N (g/kg DM)	Sediment	0.7	1.7
P (g/kg DM)	Sediment	0.2	0.5
K (g/kg DM)	Sediment	1.0	1.4
Mg (g/kg DM)	Sediment	10.2	17.6
Ca (g/kg DM)	Sediment	328.6	311.6
C/N ratio	Sediment	30	9

*Seawater was enriched with nutrients (human mistake).

Sediment/seawater interphase from Greece: Biodegradation results

Figure 120 shows the evolution of the biodegradation of the different samples in the sediment/seawater interface from Greece. The test is stopped after 226 days. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. A negative biodegradation was observed for LDPE. At the end of the test following biodegradation percentages were measured: -11.7% ± 7.1% for LDPE, 90.2% ± 84.0% for PHB copolymer, 36.9% ± 37.9% for PBSe and 15.7% ± 38.9% for PBSeT. These standard deviations clearly illustrate that the replicates behave very different. Again unrealistic values (biodegradation percentages > 100%) were measured for PHB copolymer. This indicates that the test is not reliable.

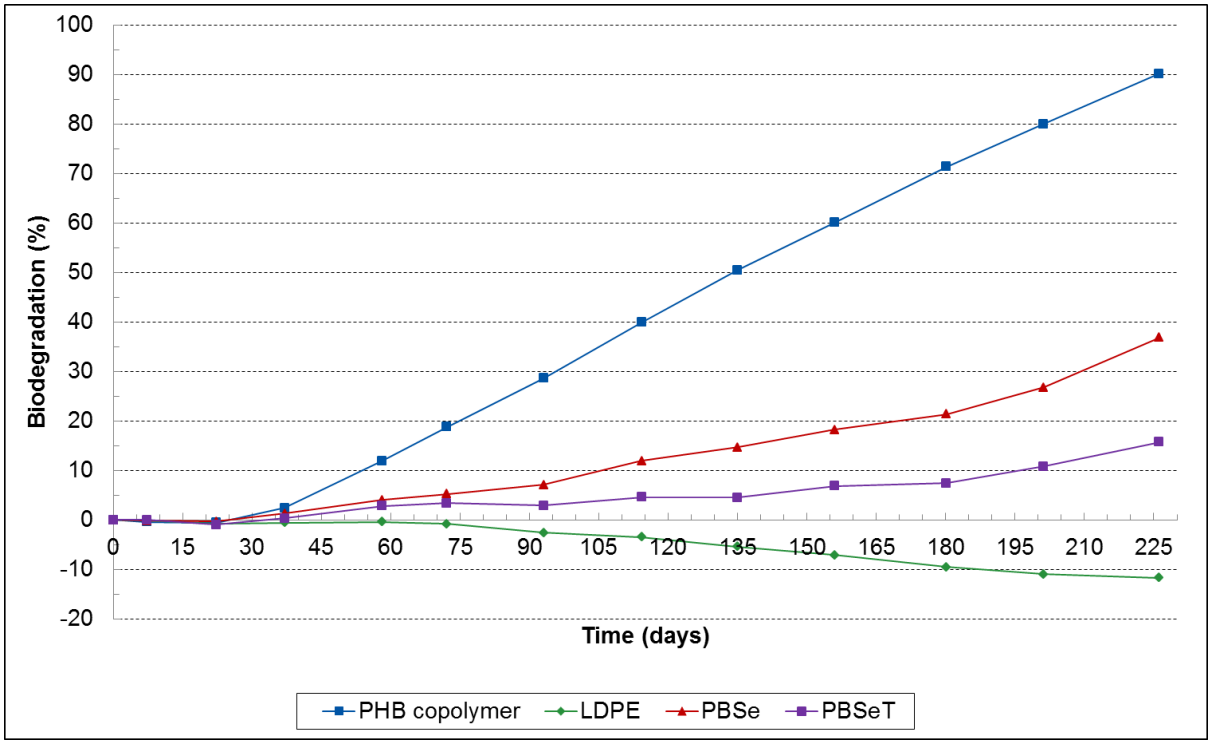


Figure 120. Evolution of biodegradation in sediment/seawater interface (Greece)

The evolution of the total CO_2 production of the individual replicates is given in Figure 121 up to Figure 125.

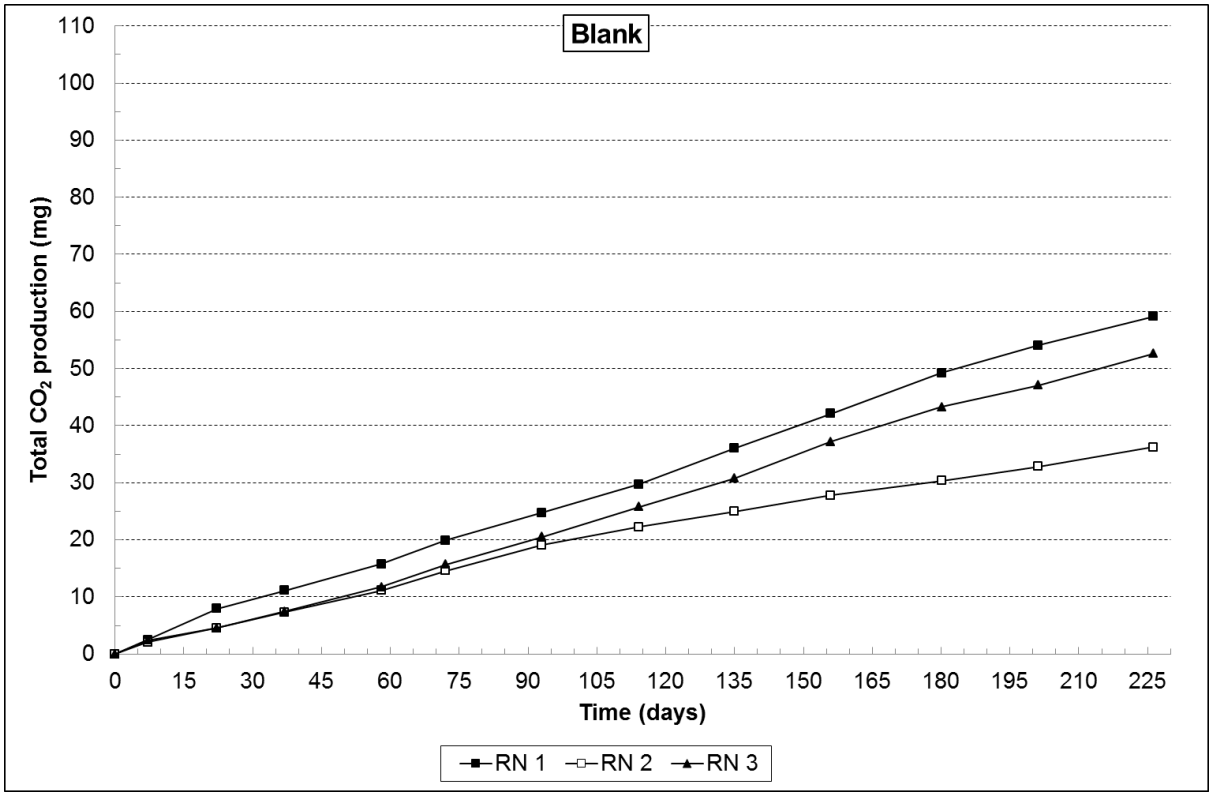


Figure 121. Total CO_2 production in blank reactors in sediment/seawater interface (Greece)

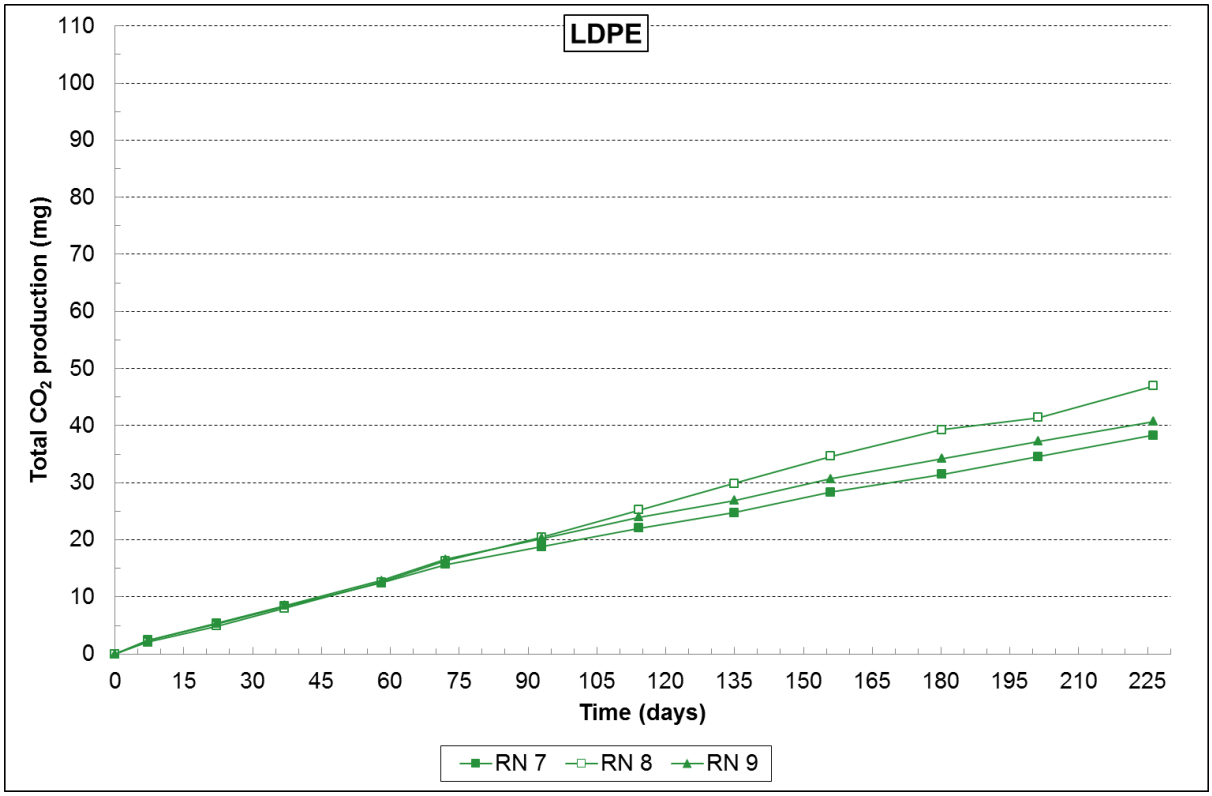


Figure 122. Total CO_2 production in LDPE reactors in sediment/seawater interface (Greece)

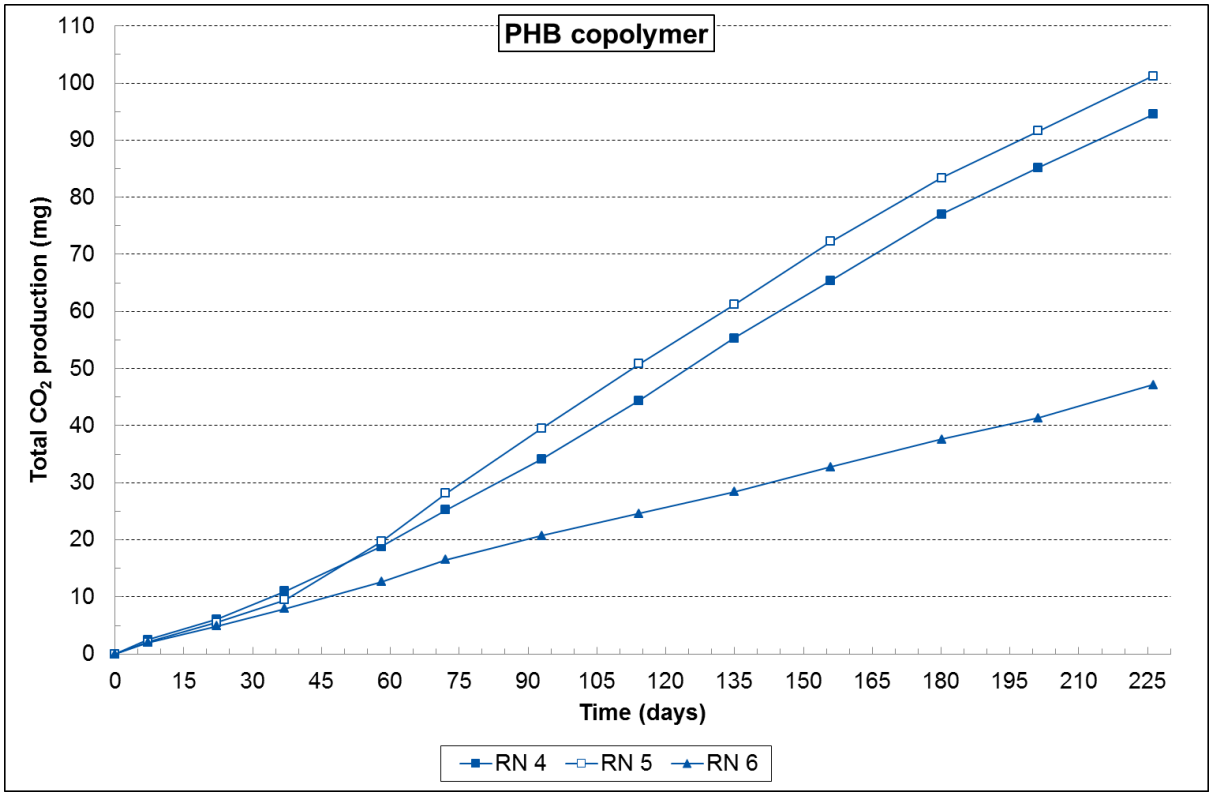


Figure 123. Total CO_2 production in PHB copolymer reactors in sediment/seawater interface (Greece)

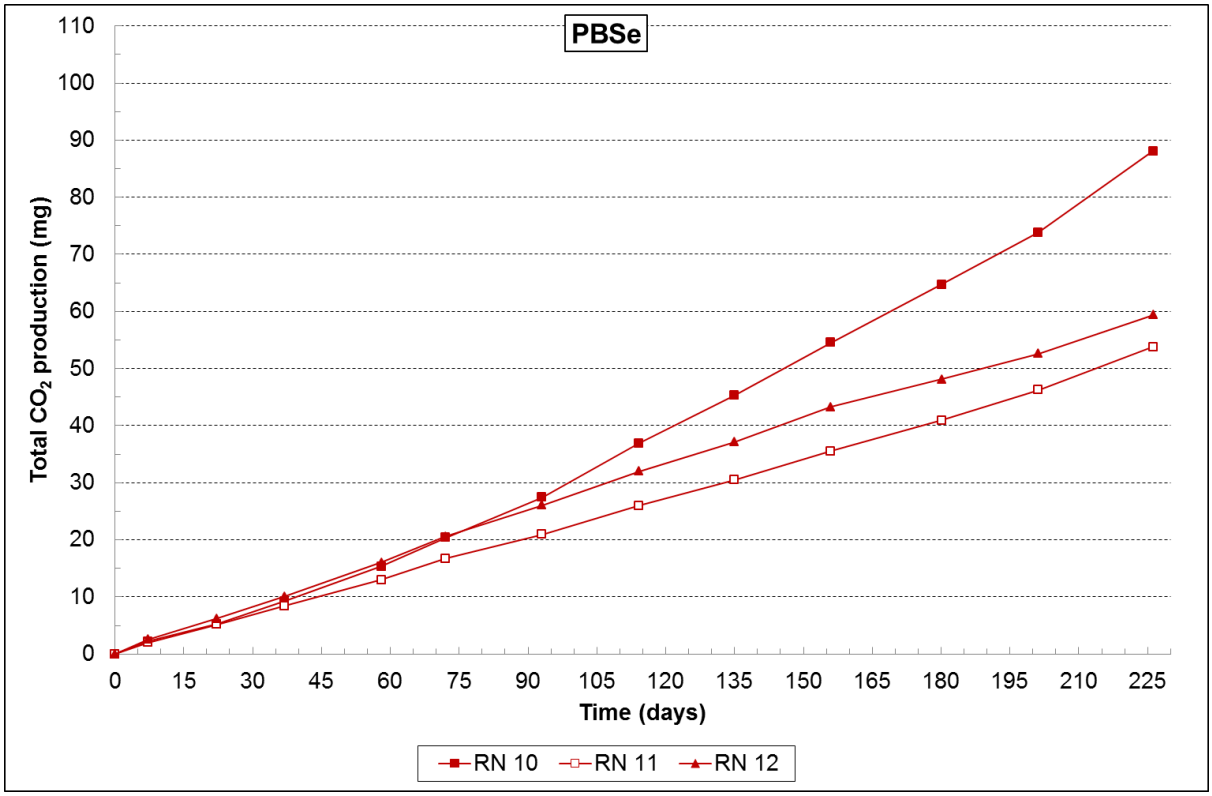


Figure 124. Total CO_2 production in PBSe reactors in sediment/seawater interface (Greece)

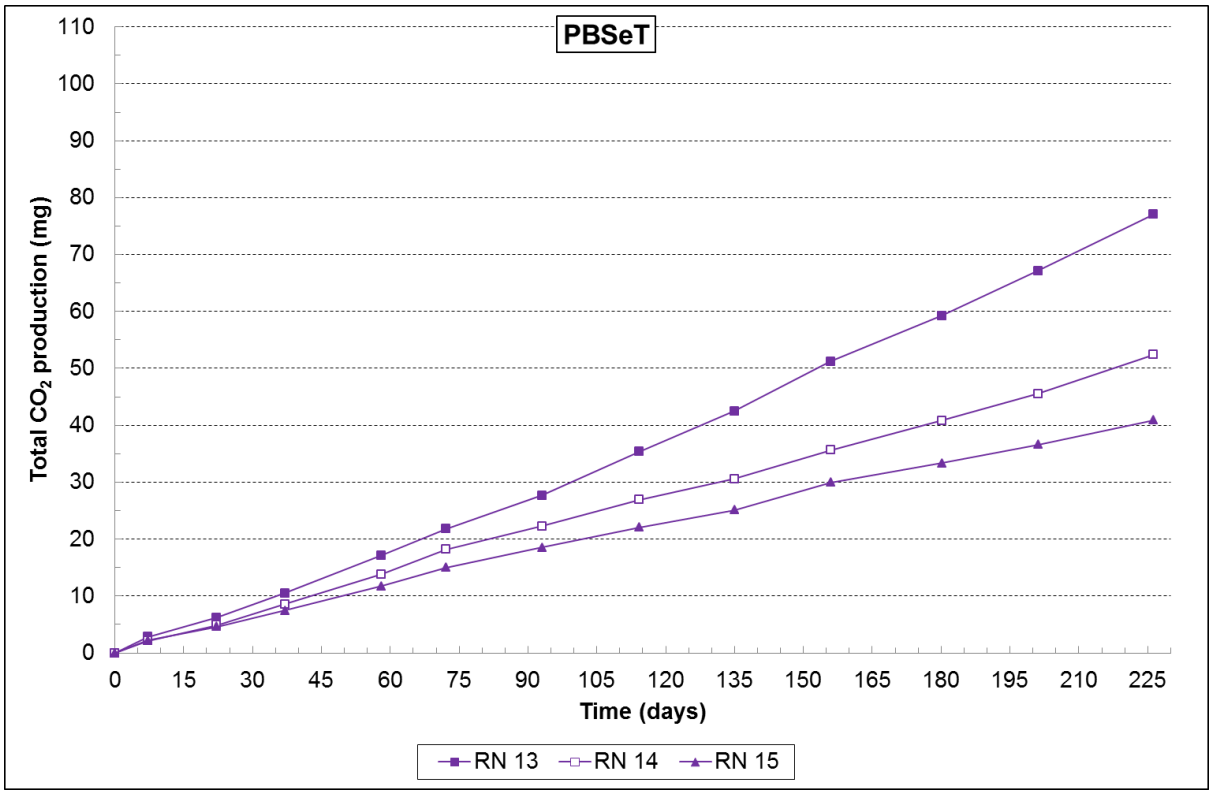


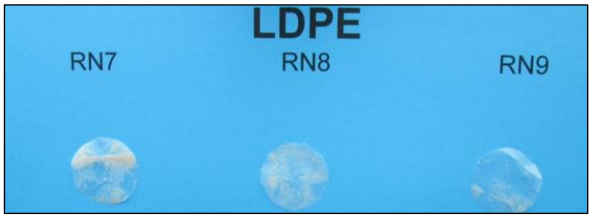
Figure 125. Total CO_2 production in PBSeT reactors in sediment/seawater interface (Greece)

At the end of the test (= after 227 days) the remaining test material was manually retrieved, dried and weighted. Table 52 gives an overview of the biodegradation percentages, the weights of the samples at start and at the end of the test and the disintegration percentages. An overview of the retrieved sample is given in Figure 126 For PHB the disintegration of the sample is in line with the biodegradation percentages (highest degree of disintegration is observed for sample with highest biodegradation percentage and lowest degree of disintegration is observed for sample with lowest biodegradation percentage). For PBSe and PBSeT no clear link is observed between the biodegradation and the disintegration percentages. However, for PBSeT it is noted that the "visual status" of the retrieved sample indeed reflects the difference in biodegradation. RN13 (58.5% biodegradation) was strongly fragmented, RN14 (6.4% biodegradation) had become brittle (tears appeared quickly in the material when mechanical stress was performed) and RN15 (-17.7% biodegradation) remained intact.

Table 52. Biodegradation and disintegration percentage of retrieved test materials after 227 days

Test item	Biodegradation (%)	Weight sample at start (mg)	Weight sample at end (mg)	Disintegration (%)
LDPE	RN7 = -17.5% RN8 = -3.8% RN9 = -13.7%	RN7 = 20.2 RN8 = 20.2 RN9 = 20.2	RN7 = 22.8 RN8 = 21.9 RN9 = 21.3	RN7 =* RN8 = * RN9 = *
PHB copolymer	RN4 = 129.5% RN5 = 147.3% RN6 = -6.2%	RN4 = 19.9 RN5 = 20.1 RN6 = 20.0	RN4 = 12.8 RN5 = 4.0 RN6 = 19.8	RN4 = 35.7 RN5 = 80.1 RN6 = 1.0
PBSe	RN10 = 80.1% RN11 = 9.3% RN12 = 21.2%	RN10 = 20.2 RN11 = 20.2 RN12 = 19.8	RN10 = 53.3 RN11 = 34.5 RN12 = 50.2	RN10 = * RN11 = * RN12 = *
PBSeT	RN13 = 58.5% RN14 = 6.4% RN15 = -17.7%	RN13 = 19.8 RN14 = 20.0 RN15 = 19.9	RN13 = 20.1 RN14 = 29.2 RN15 = 23.8	RN13 = * RN14 = * RN15 = *

* weight of the retrieved sample at the end of the test is higher than the weight of the sample at start



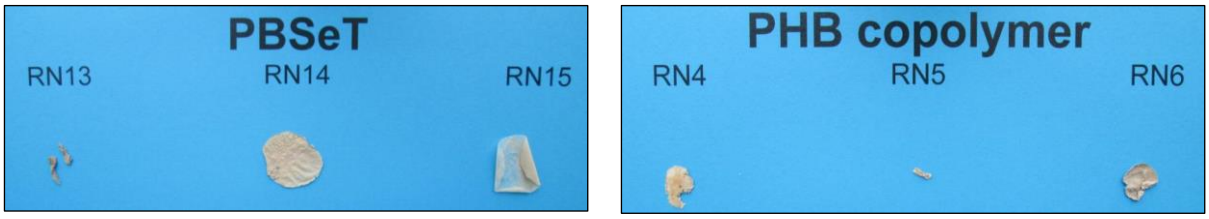


Figure 126. Overview of retrieved pieces of LDPE, PBSe, PBSeT and PHB in sediment/seawater interface (Greece)

Sediment/seawater interphase from Italy: Biodegradation results

Figure 127 shows the evolution of the biodegradation of the different samples in the sediment/seawater interface from Italy. The test is stopped after 180 days. Negative biodegradation percentages were obtained for all samples expect for PHB copolymer. After 180 days following biodegradation percentages were measured: -27.4% ± 23.0% for LDPE, 37.2% ± 26.8% for PHB copolymer, -25.6% ± 26.5% for PBSe and -46.2% ± 5.8% for PBSeT. For one replicate of the PHB copolymer series the biodegradation was significantly lagging behind. The biodegradation values of the three replicates after 180 days were 63.4%, 9.9% and 38.2%.

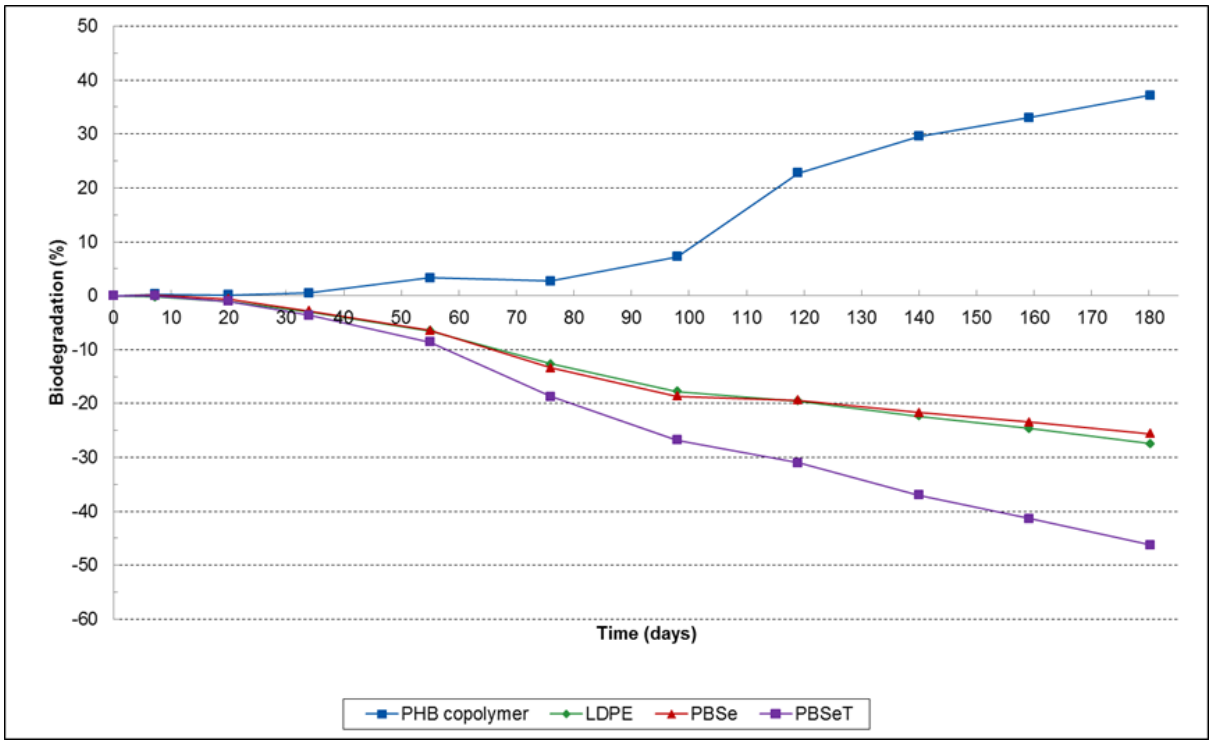


Figure 127. Evolution of biodegradation in sediment/seawater interface (Italy)

The evolution of the total CO_2 production of the individual replicates is given in Figure 128 up to Figure 132.

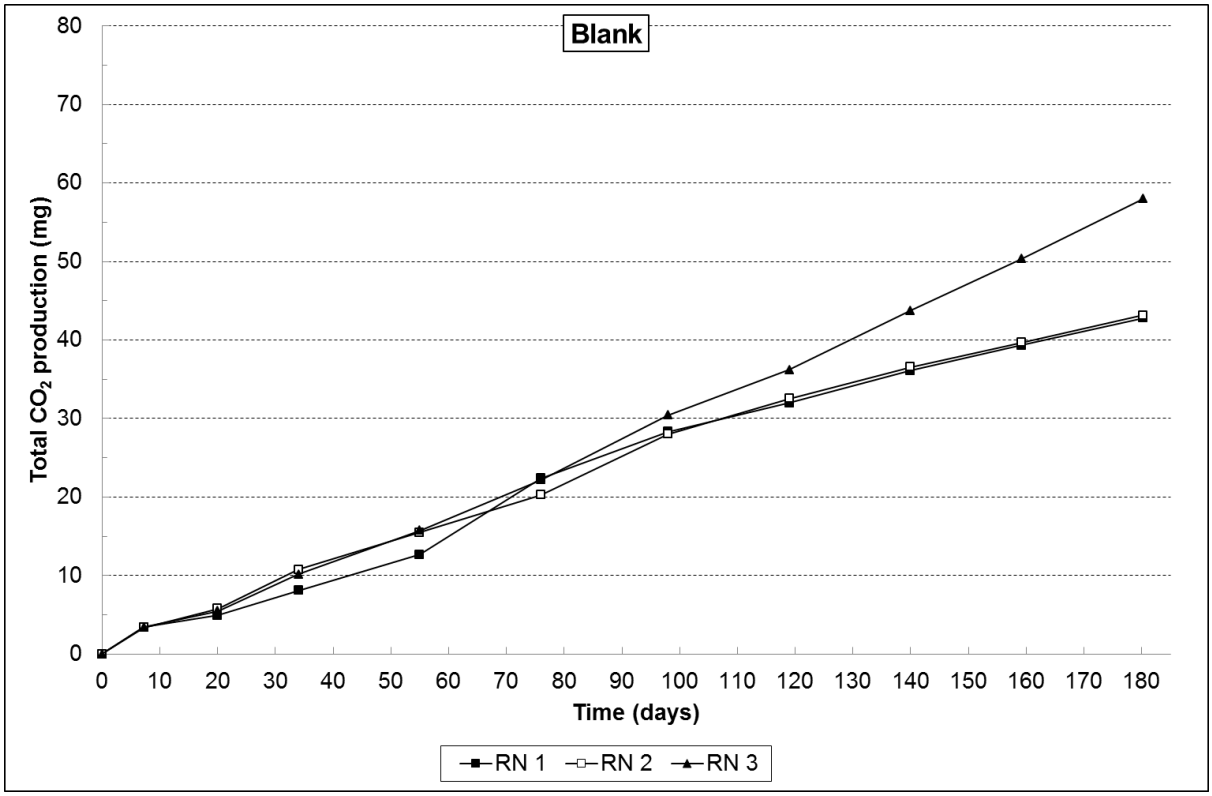


Figure 128. Total CO_2 production in blank reactors in sediment/seawater interface (Italy)

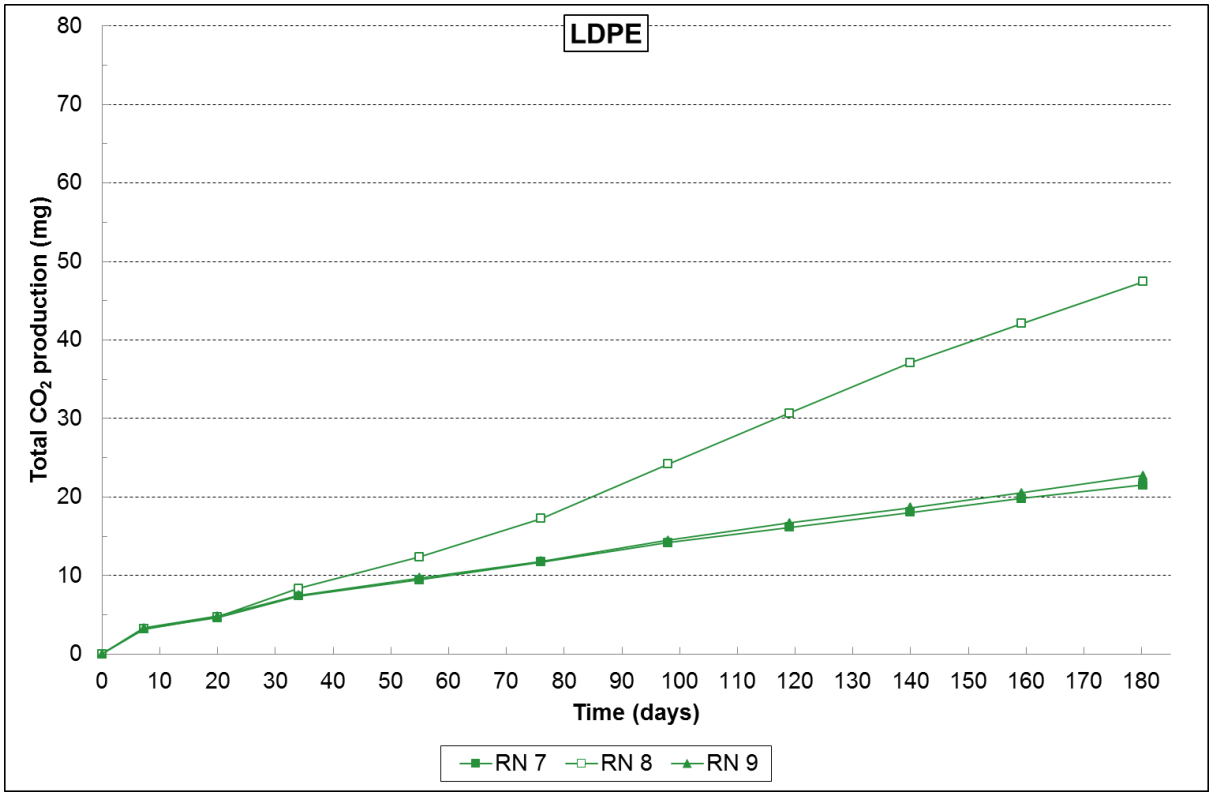


Figure 129. Total CO_2 production in LDPE reactors in sediment/seawater interface (Italy)

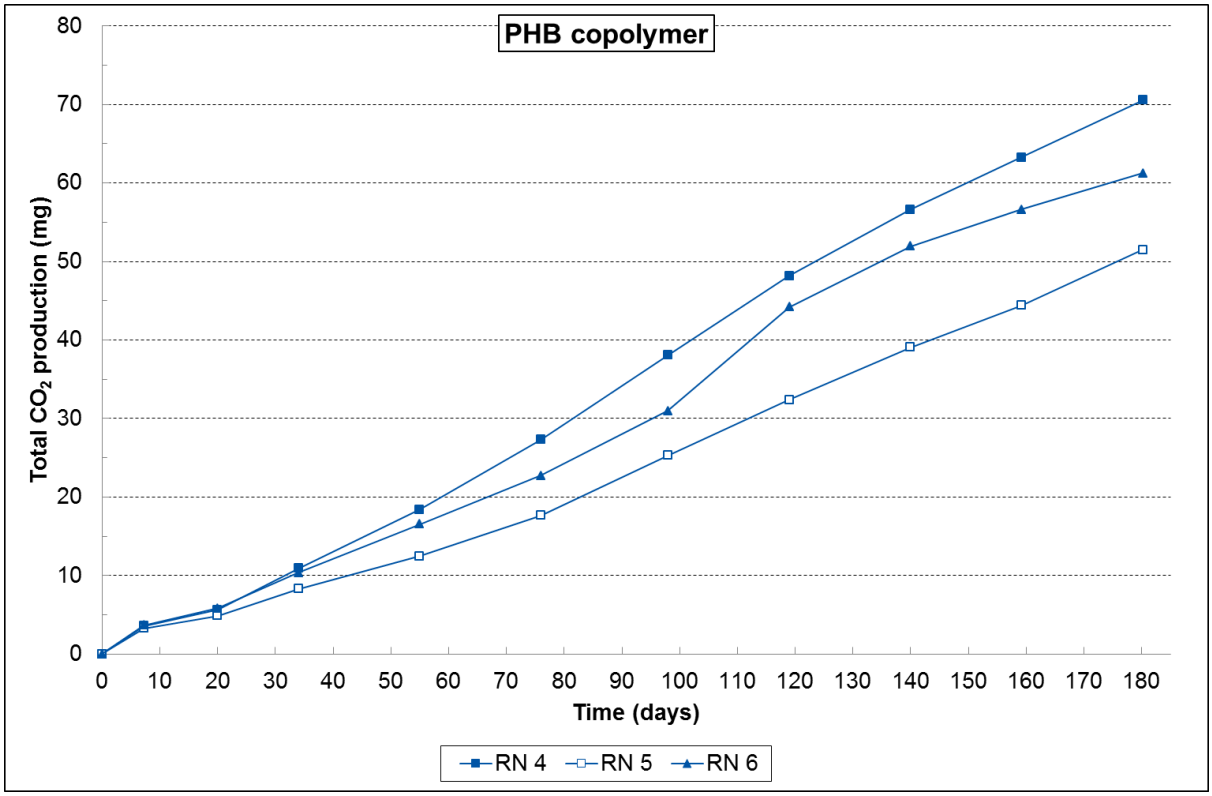


Figure 130. Total CO_2 production in PHB copolymer reactors in sediment/seawater interface (Italy)

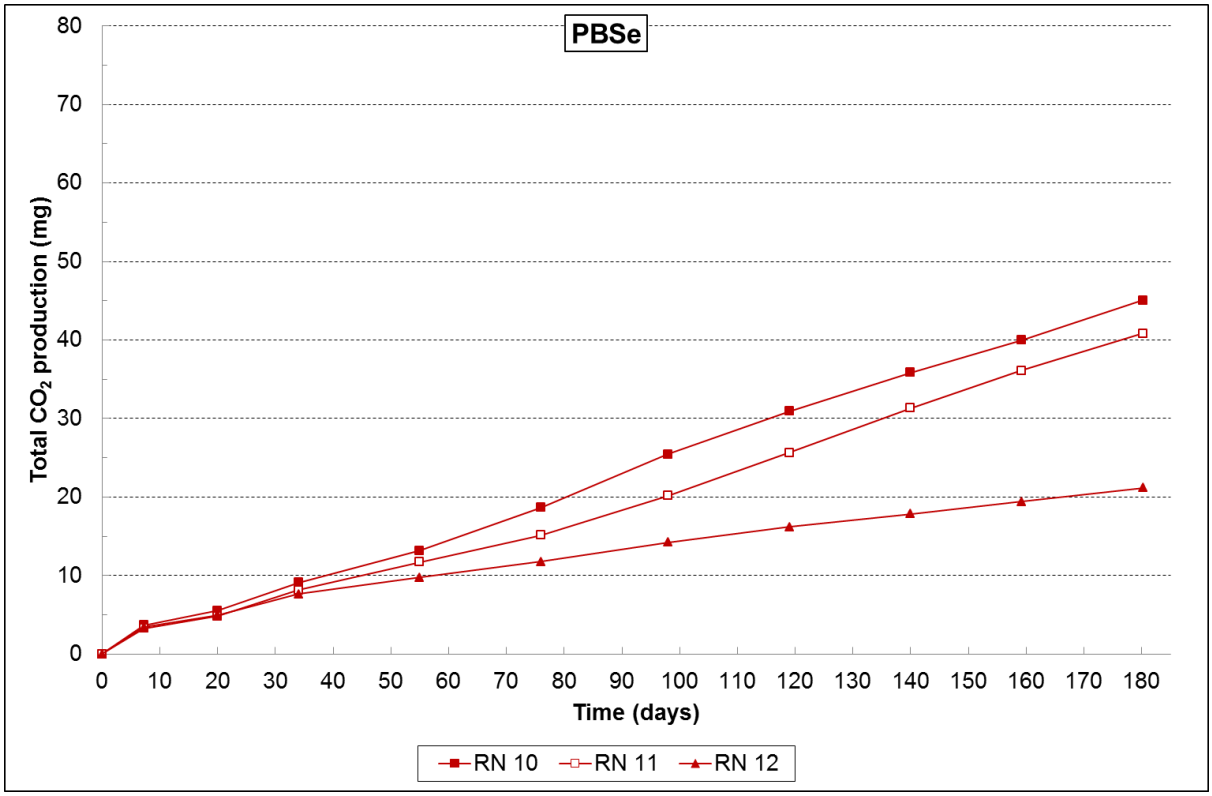


Figure 131. Total CO_2 production in PBSe reactors in sediment/seawater interface (Italy)

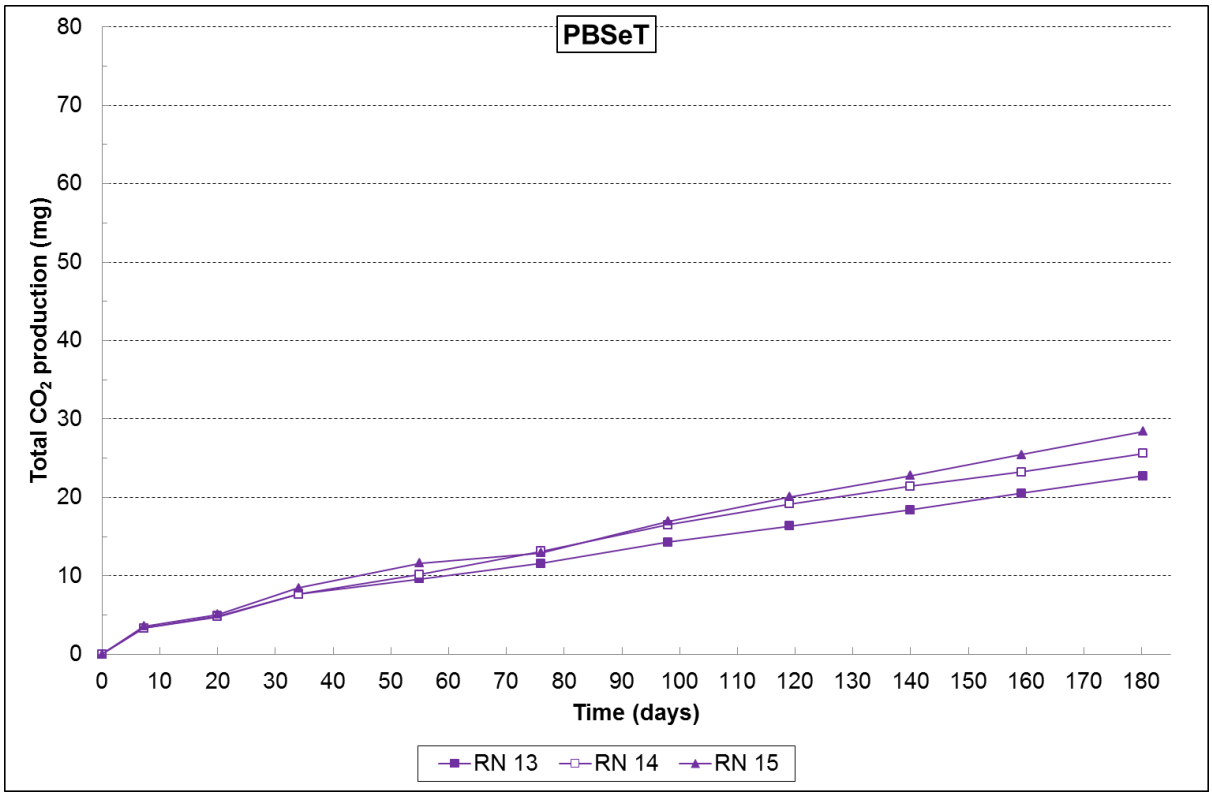


Figure 132. Total CO_2 production in PBSeT reactors in sediment/seawater interface (Italy)

Table 53. Description and disintegration percentage of retrieved test materials after 180 days

Test item	Weight sample at start (mg)	Weight sample at end (mg)	Disintegration (%)
LDPE	RN7 = 20.3 RN8 = 20.2 RN9 = 20.5	RN7 = 22.8 RN8 = 22.2 RN9 = 23.7	RN7 = ** RN8 = ** RN9 =**
PHB copolymer	RN4 = 20.3 RN5 = 20.3 RN6 = 19.8	RN4 = n.r. RN5 = 18.3 RN6 = n.r.	RN4 = 100% RN5 = 10% RN6 = 100%
PBSe	RN10 = 20.2 RN11 = 20.0 RN12 = 20.1	RN10 = 22.1 RN11 = 24.0 RN12 = 21.2	RN10 = ** RN11 = ** RN12 = **
PBSeT	RN13 = 20.3 RN14 = 20.2 RN15 = 20.3	RN13 = 21.2 RN14 = 21.0 RN15 = 21.6	RN13 = ** RN14 = ** RN15 = **

* n.r. = sample not recoverable; ** weight of the sample at the end of the test is higher than the weight of the sample at start

At the end of the test (= after 180 days) the remaining test material was manually retrieved, dried and weighted. Table 53 gives an overview of the weights at start and at end of the test and the disintegration percentage. An overview of the retrieved sample is given in Figure 133. Replicate RN5 of PHB copolymer was also characterized by the lowest biodegradation.

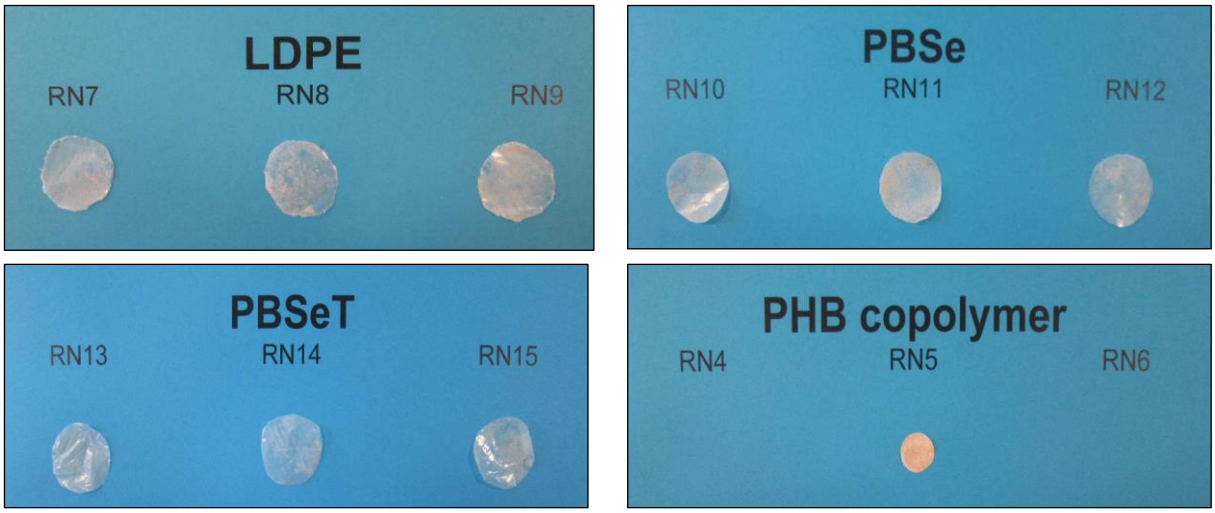


Figure 133. Overview of retrieved pieces of LDPE, PBSe, PBSeT and PHB in sediment/seawater interface (Italy)

Conclusions and recommendations

The results of the performed tests in year 1 and year 2 are not reliable. The obtained unrealistic biodegradation percentages are clearly an overestimation of the biodegradation of the samples in year 1, while in year 2 negative values are observed for several samples. The addition of 50% sand after calcination has clearly not solved the problem. These problems can be caused by anaerobic conditions in the sediment. As the sample is strongly contaminated with small sand particles at the end of the test, it is difficult/impossible to determine the disintegration of the sample accurately.

Some suggestions that could be investigated:

- Aeration of the system
- Larger reactors (thin layer of sediment on the bottom of the reactors in order to try to avoid anaerobic conditions)
- Higher sample quantity
- Addition of nutrients

In this test method, the use of negative reference material LDPE should be obliged.

6.2.3 Biodegradation in Pelagic zone

Characteristics of seawater

The results of the analyses executed on the seawater are given in Table 54 and also in Table 37

Table 54. Characteristics of the seawaters

Parameters	Greece	Italy
EC (µS/cm)	58700	56000
pH	7.4	7.2
Total N (mg/L)	45	69
P (mg/kg DM)	16	27
K (mg/kg DM)	492	461
Ca (mg/kg DM)	397	271
Mg (mg/kg DM)	1360	1315
NH_4^+-N (mg/L)	16	15

NO_x^-N (mg/L)	< 10	< 10

0.05 g NH_4Cl and 0.1 g KH_2PO_4 were added per liter seawater.

Figure 134 shows the evolution of the biodegradation of the different samples in the seawater from Greece. The test was stopped after 271 days. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. Negligible biodegradation was observed for LDPE. After 271 days following biodegradation percentages were measured: -3.1% ± 1.6% for LDPE, 74.6% ± 0.8% for PHB copolymer, 79.2% ± 6.2% for PBSe and 31.1% ± 39.1% for PBSeT. A very significant difference was observed between the different replicates of PBSeT. One replicate started after 83 days, a second replicate started after 110 days and the biodegradation of the third replicate didn't started at all. The biodegradation values of the three replicates after 271 days were -4.4%, 73.1% and 24.6% (Figure 135).

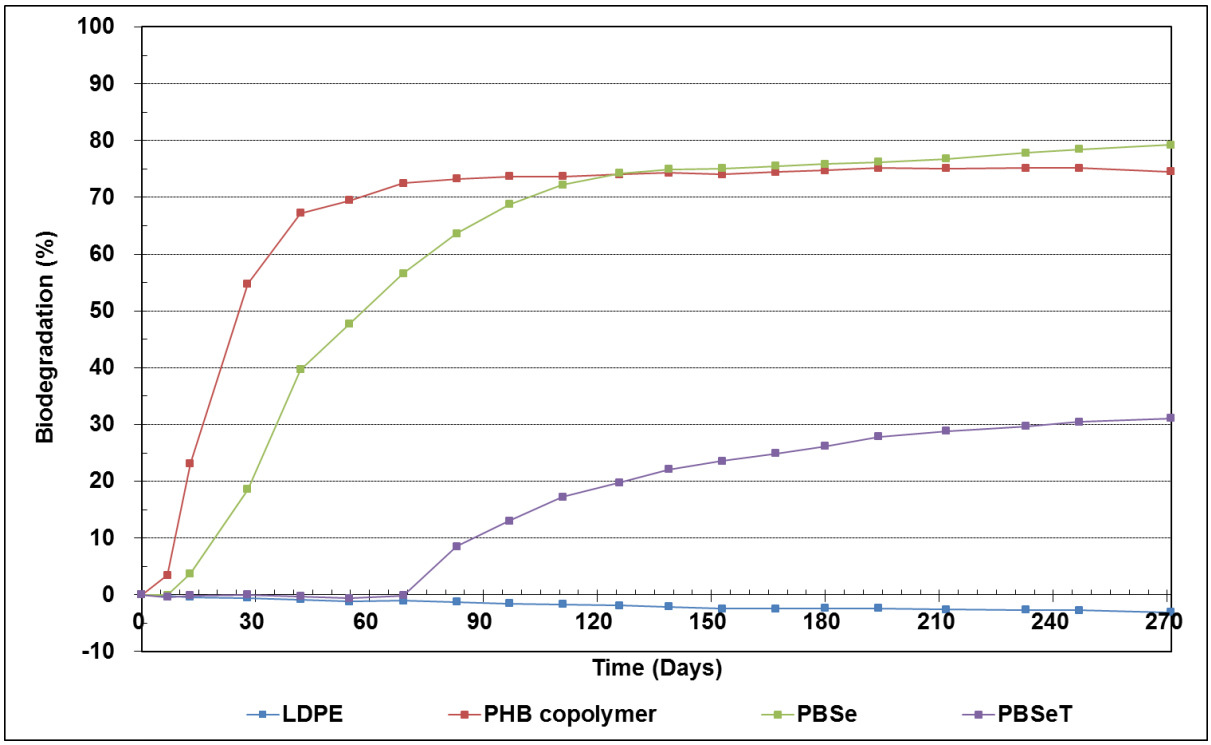


Figure 134. Evolution of biodegradation in seawater (Greece)

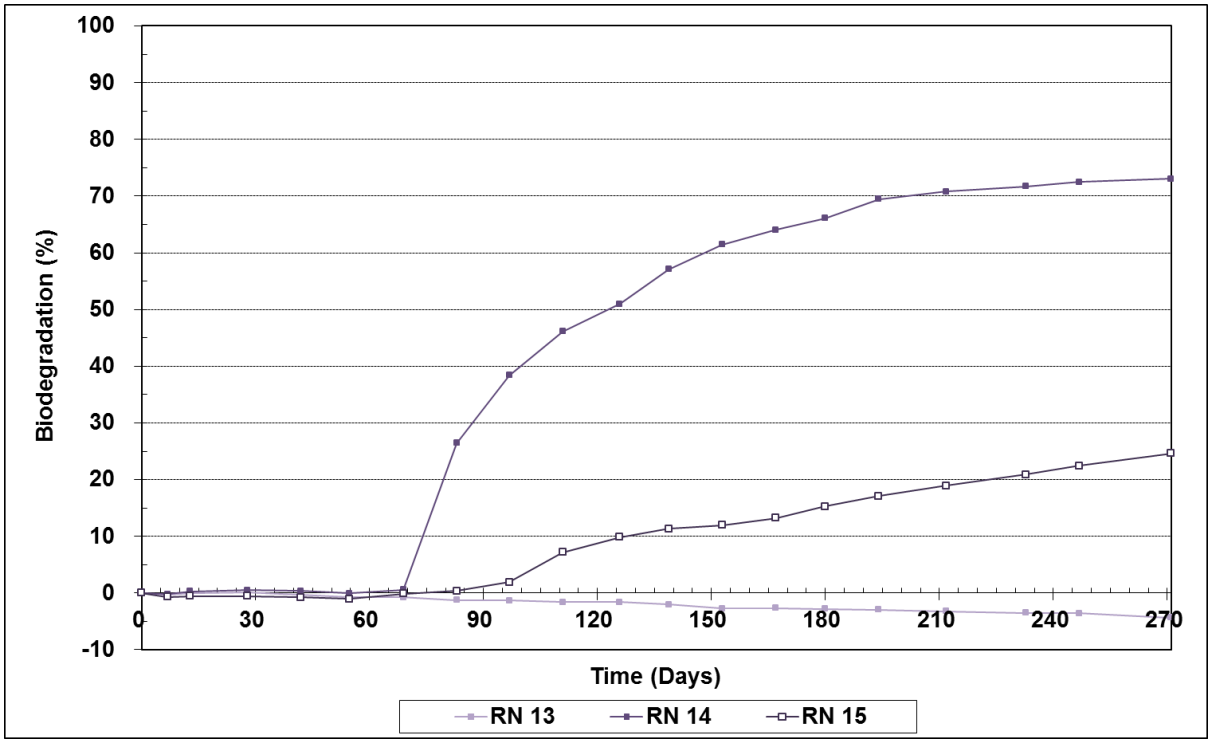


Figure 135. Evolution of biodegradation of different replicates of PBSeT in seawater (Greece)

The evolution of the total CO_2 production of the individual replicates is given in Figure 136 up to Figure 140.

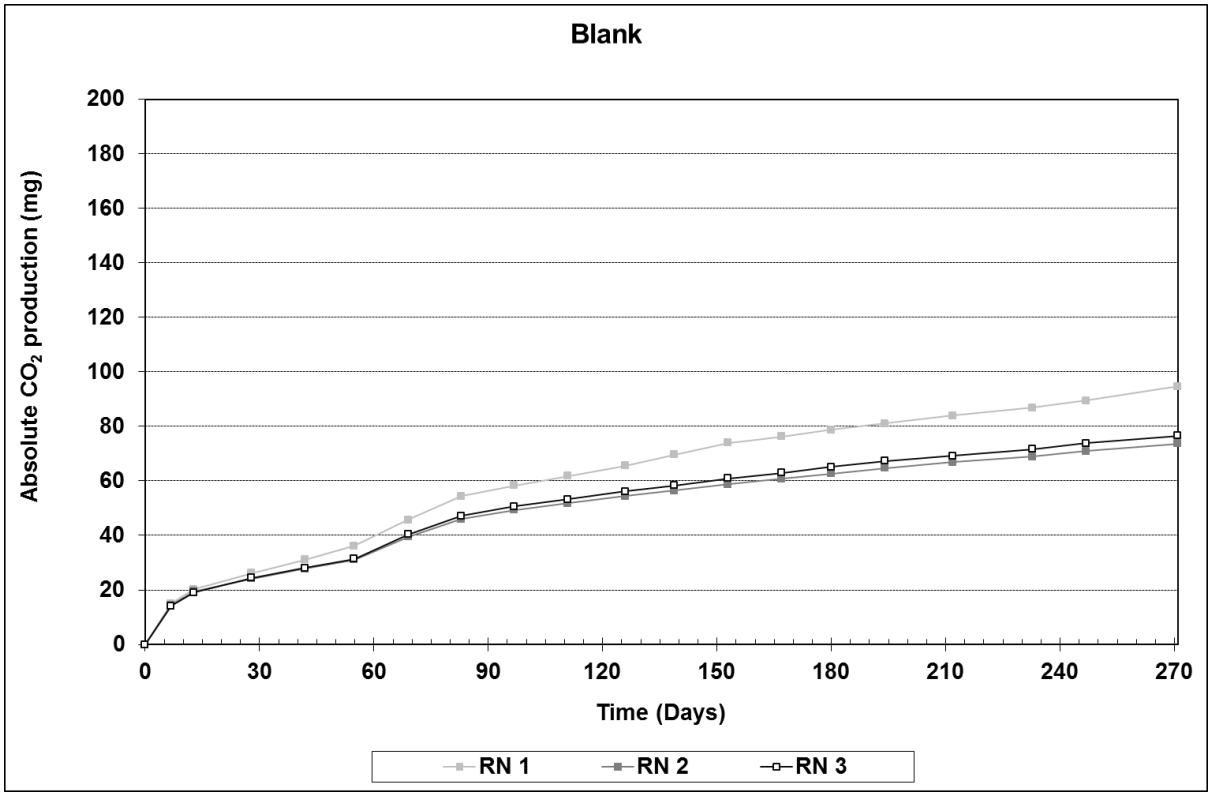


Figure 136. Total CO_2 production in blank reactors in seawater (Greece)

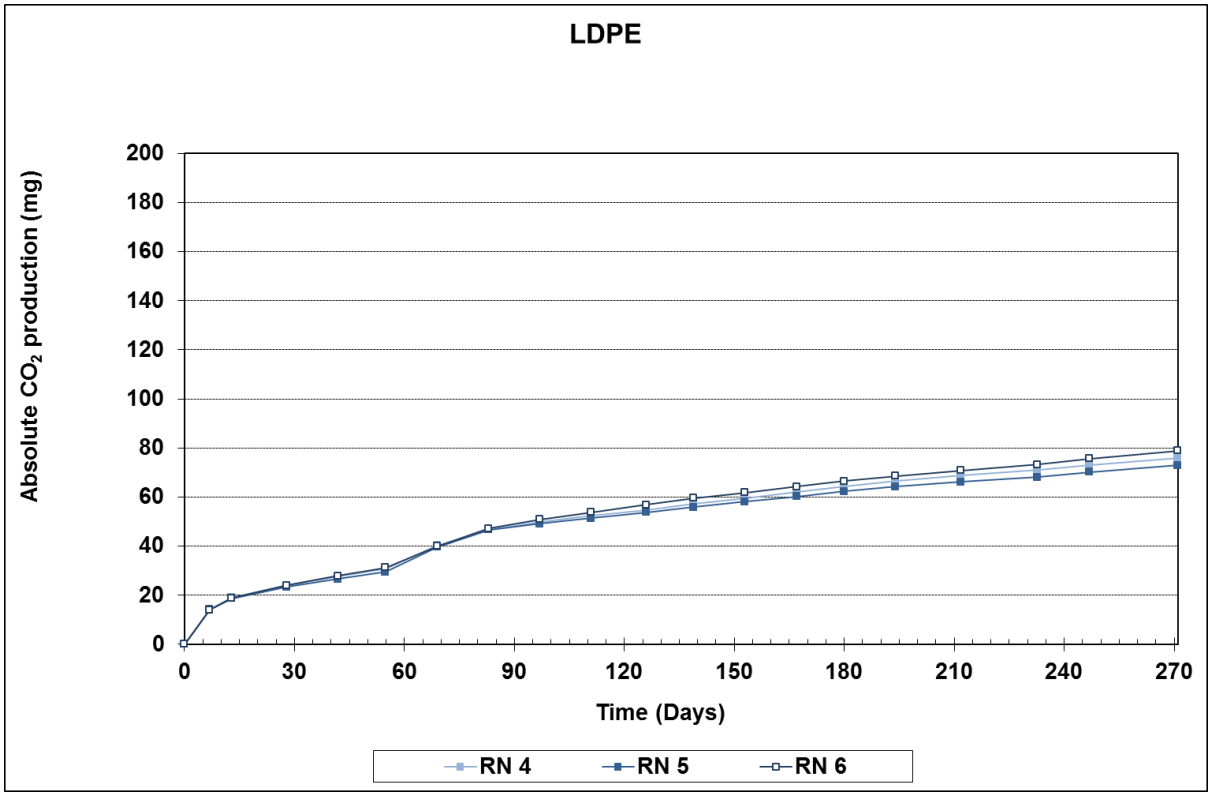


Figure 137. Total CO_2 production in LDPE reactors in seawater (Greece)

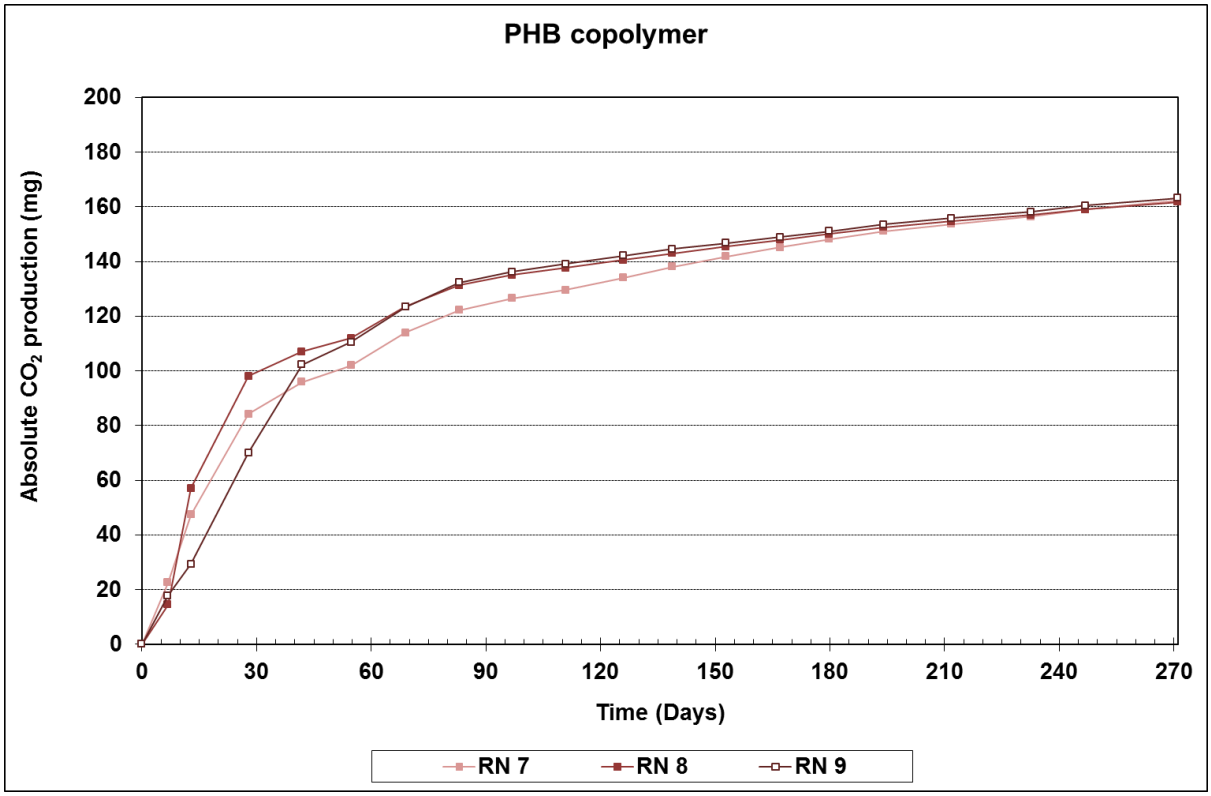


Figure 138. Total CO_2 production in PHB copolymer reactors in seawater (Greece)

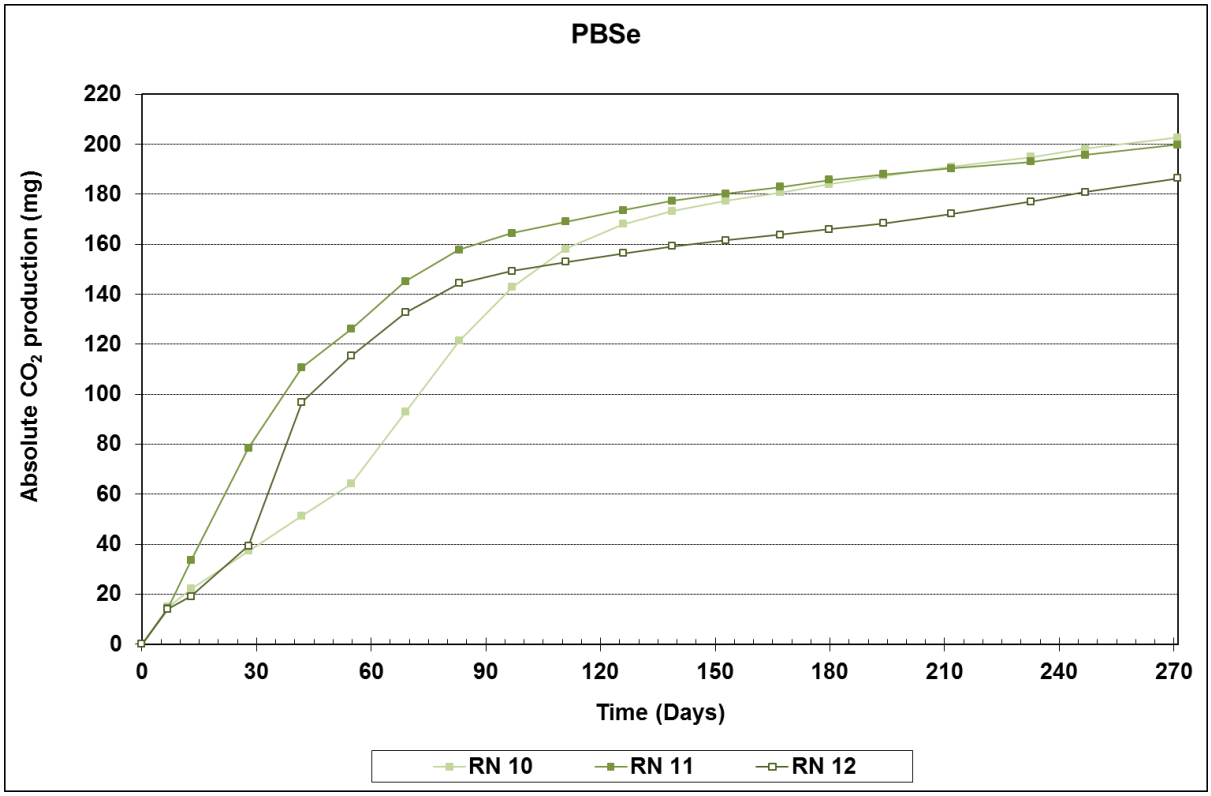


Figure 139. Total CO_2 production in PBSe reactors in seawater (Greece)

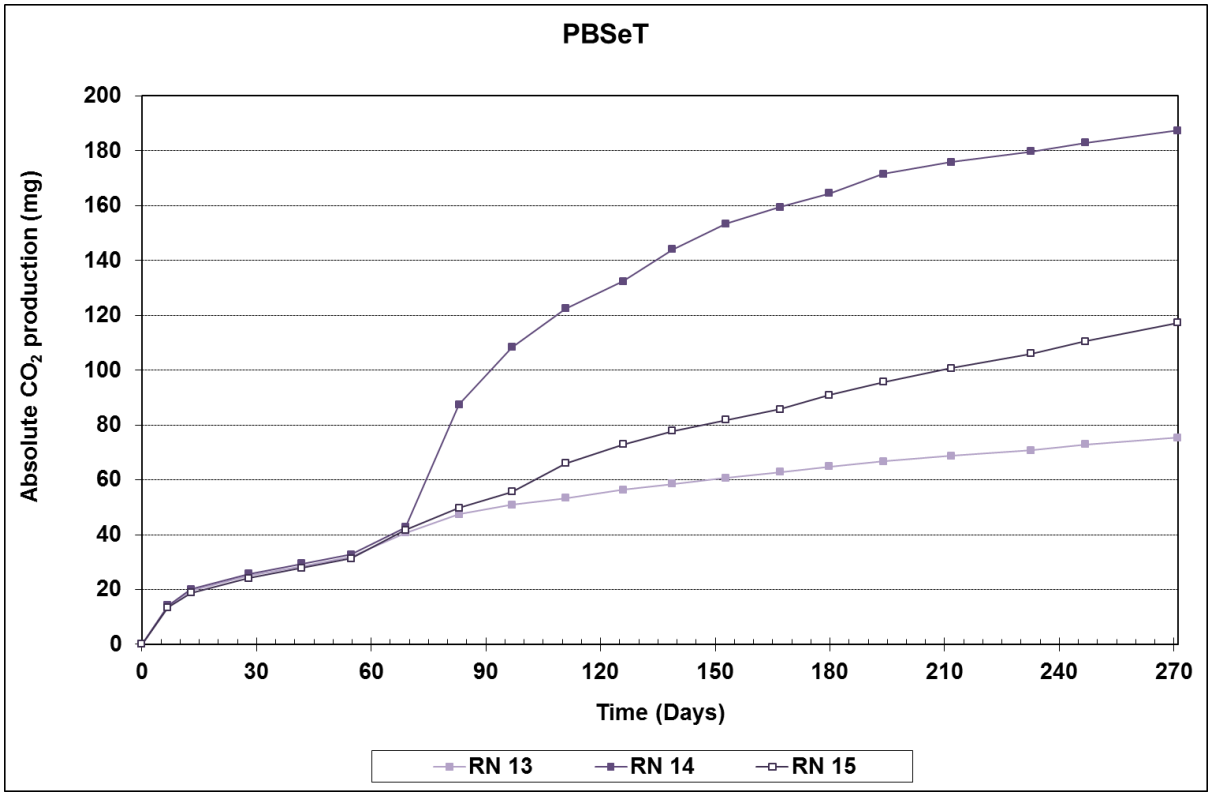


Figure 140. Total CO_2 production in PBSeT reactors in seawater (Greece)

Figure 141 shows the evolution of the biodegradation of the different samples in the seawater from Italy. The test was stopped after 180 days. As oxitop measuring heads were available, also the oxygen consumption was measured simultaneously (Figure 142). The biodegradation percentages are somewhat higher when compared to the biodegradation percentages based on carbon dioxide production, but the trend of the biodegradation is very similar. The difference in biodegradation pattern between the samples is logical. The fastest biodegradation is observed for PHB copolymer, followed by PBSe and PBSeT. It is noted that PBSe reaches a higher biodegradation level when compared to PHB. Negligible biodegradation was observed for LDPE. At the end of the test (= after 180 days) following biodegradation percentages were measured: -0.7% ± 3.3% for LDPE, 72.2% ± 1.7% for PHB copolymer, 92.3% ± 18.6% for PBSe and 78.4% ± 13.0% for PBSeT. The variability between the replicates is rather acceptable. Only for PBSe some variation was observed between the different replicates (standard deviation: 13%). One of the PBSe replicates is characterized by an unrealistic high value (112.9% after 180 days). Not taken into account this high value, PBSe would reach a plateau at a level of 82.0%.

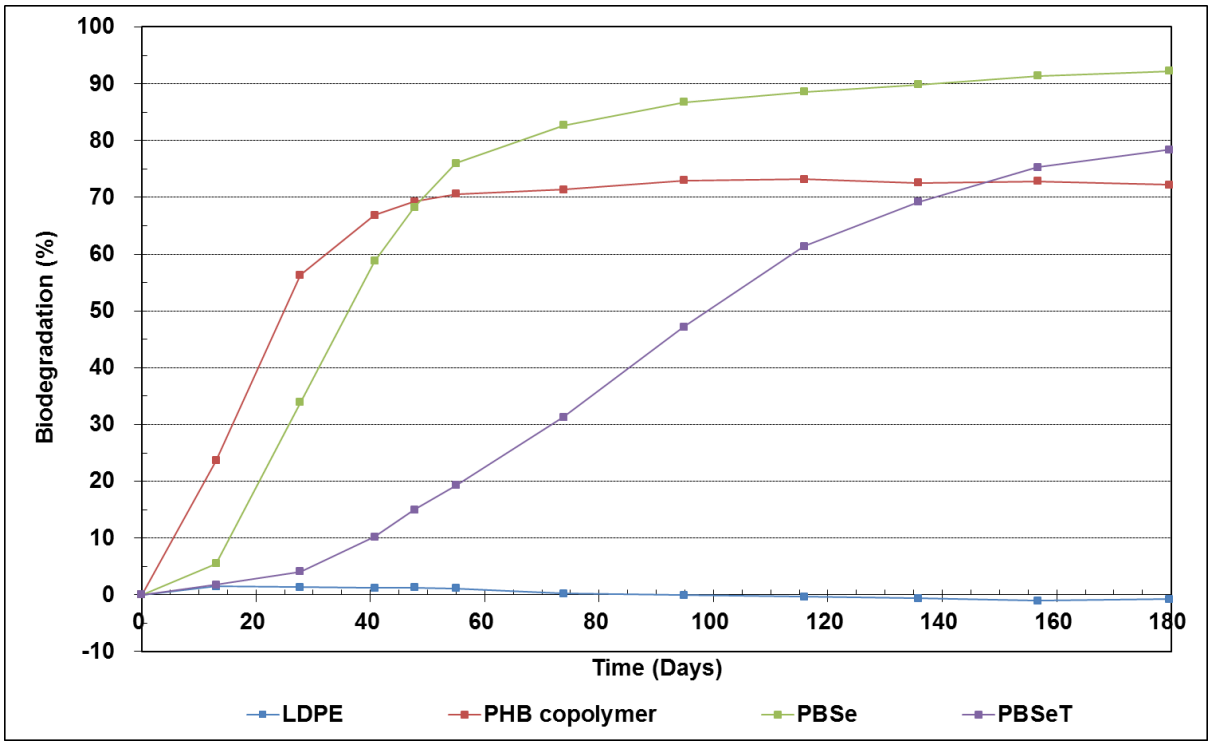


Figure 141. Evolution of biodegradation in seawater (Italy) (based on carbon dioxide production)

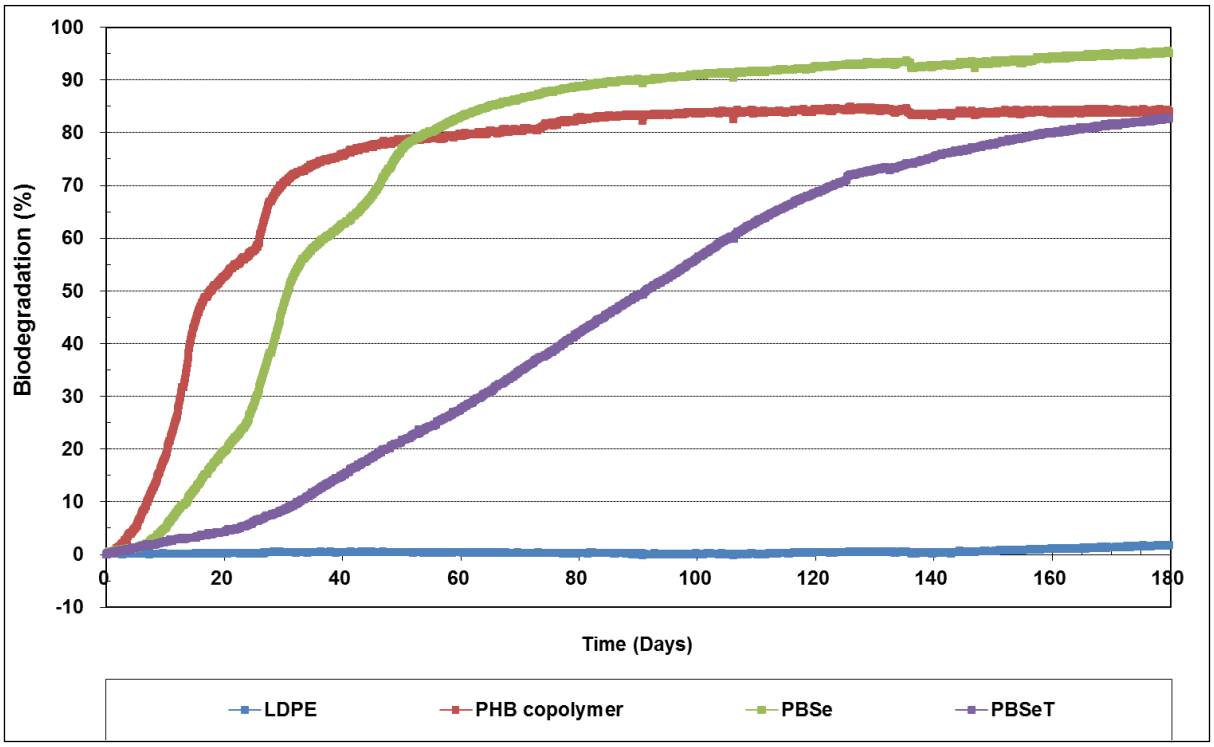


Figure 142. Evolution of biodegradation in seawater (Italy) (based on oxygen consumption)

The evolution of the total CO_2 production of the individual replicates is given in Figure 143 up to Figure 147.

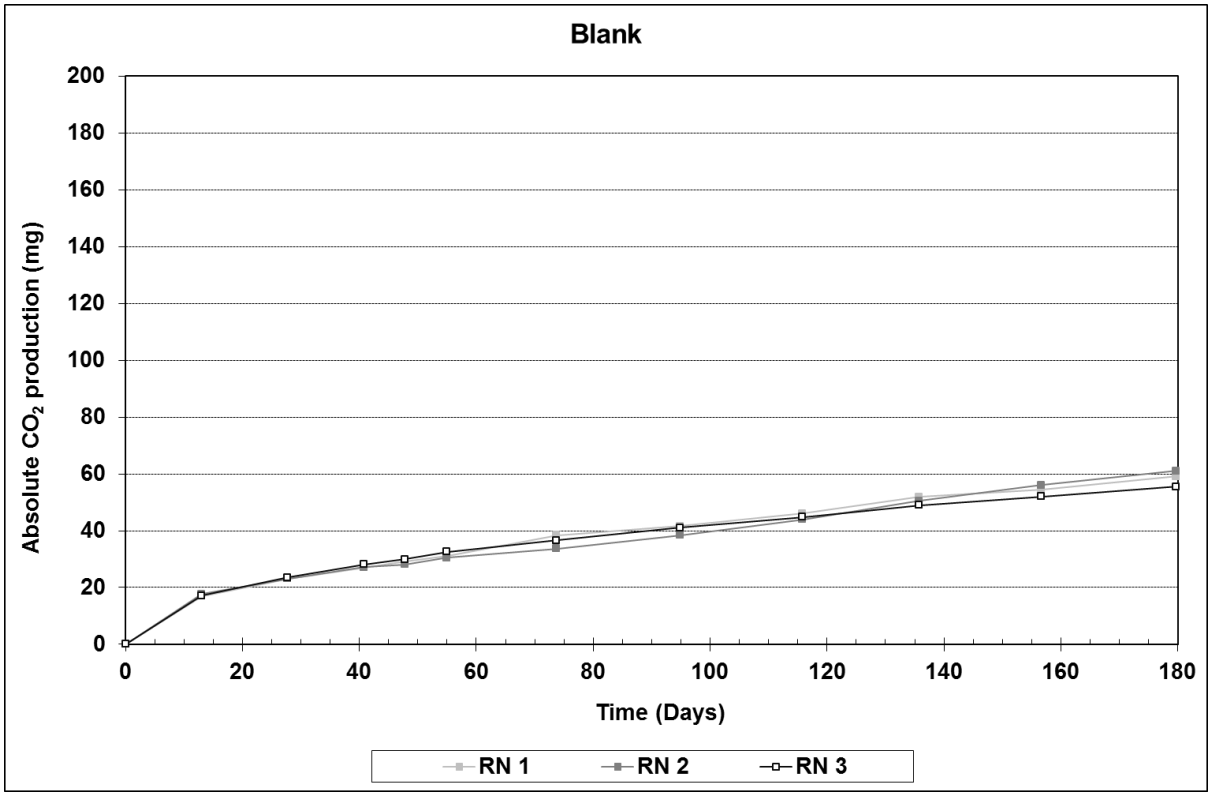


Figure 143. Total CO_2 production in blank reactors in seawater (Italy)

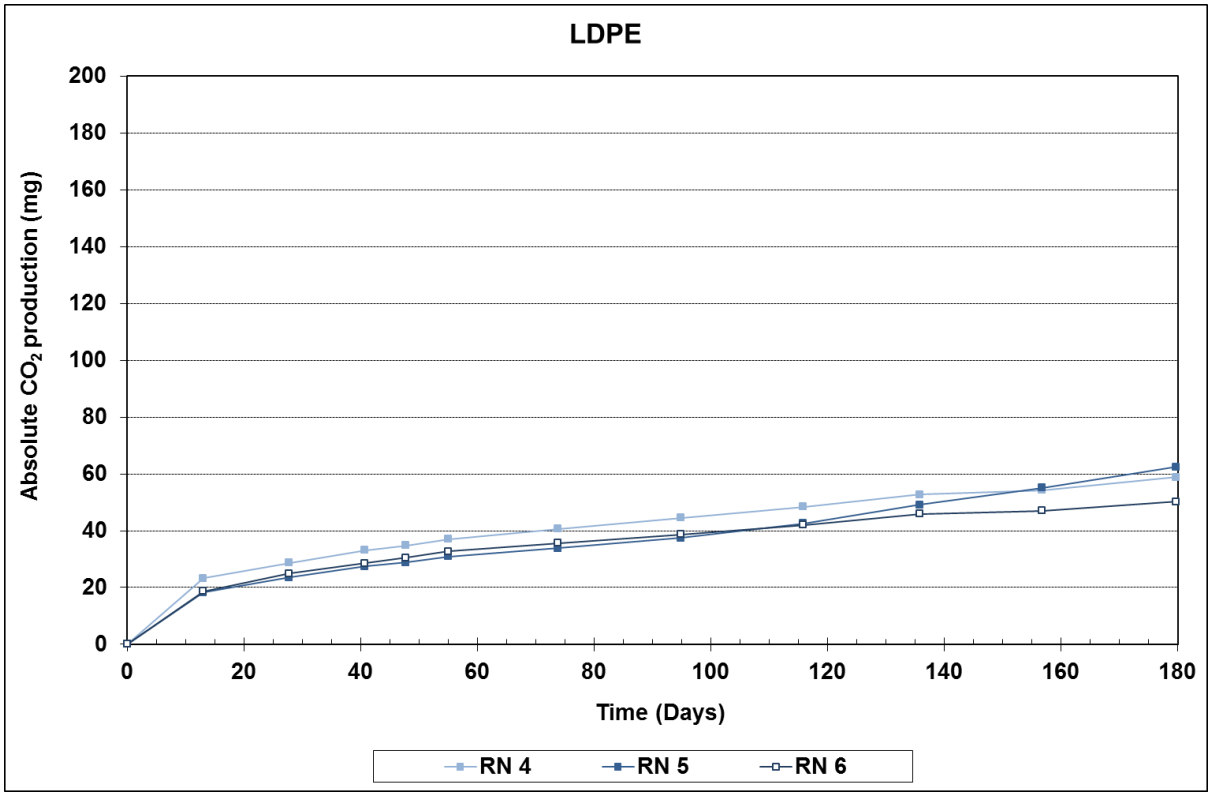


Figure 144. Total CO_2 production in LDPE reactors in seawater (Italy)

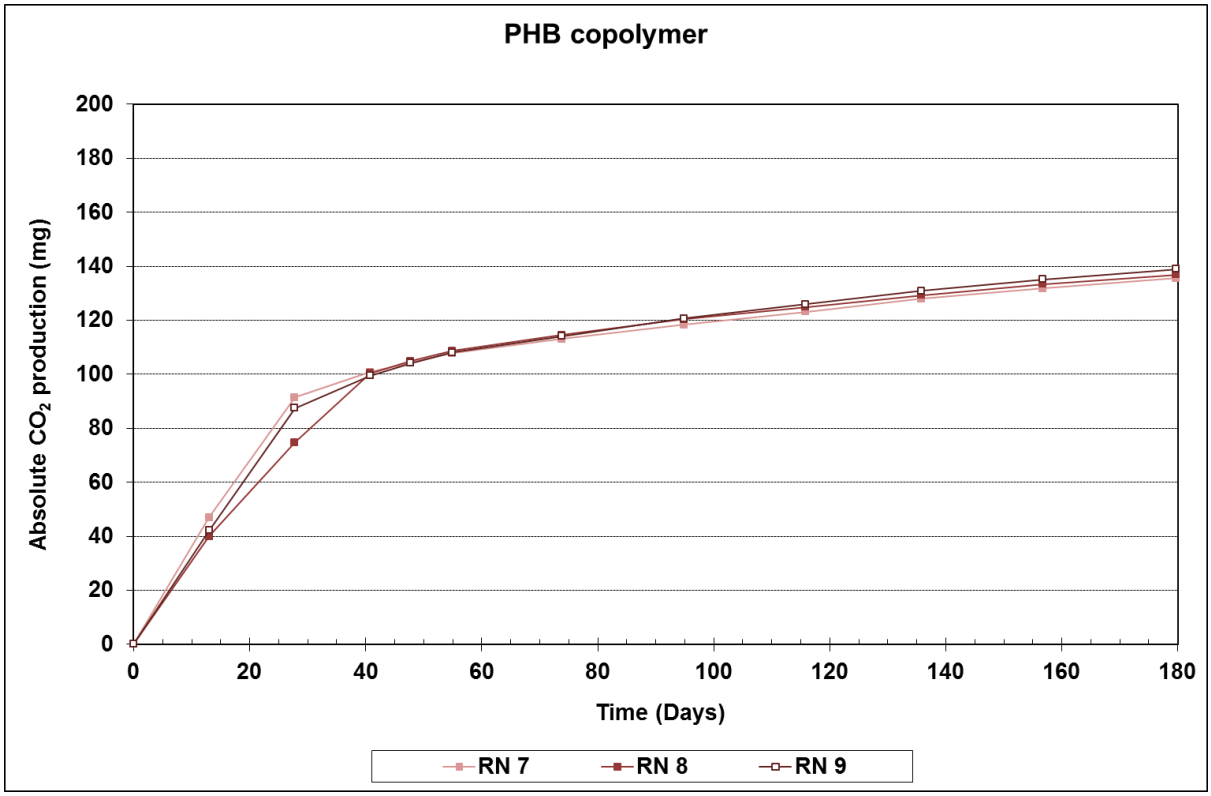


Figure 145. Total CO_2 production in PHB copolymer reactors in seawater (Italy)

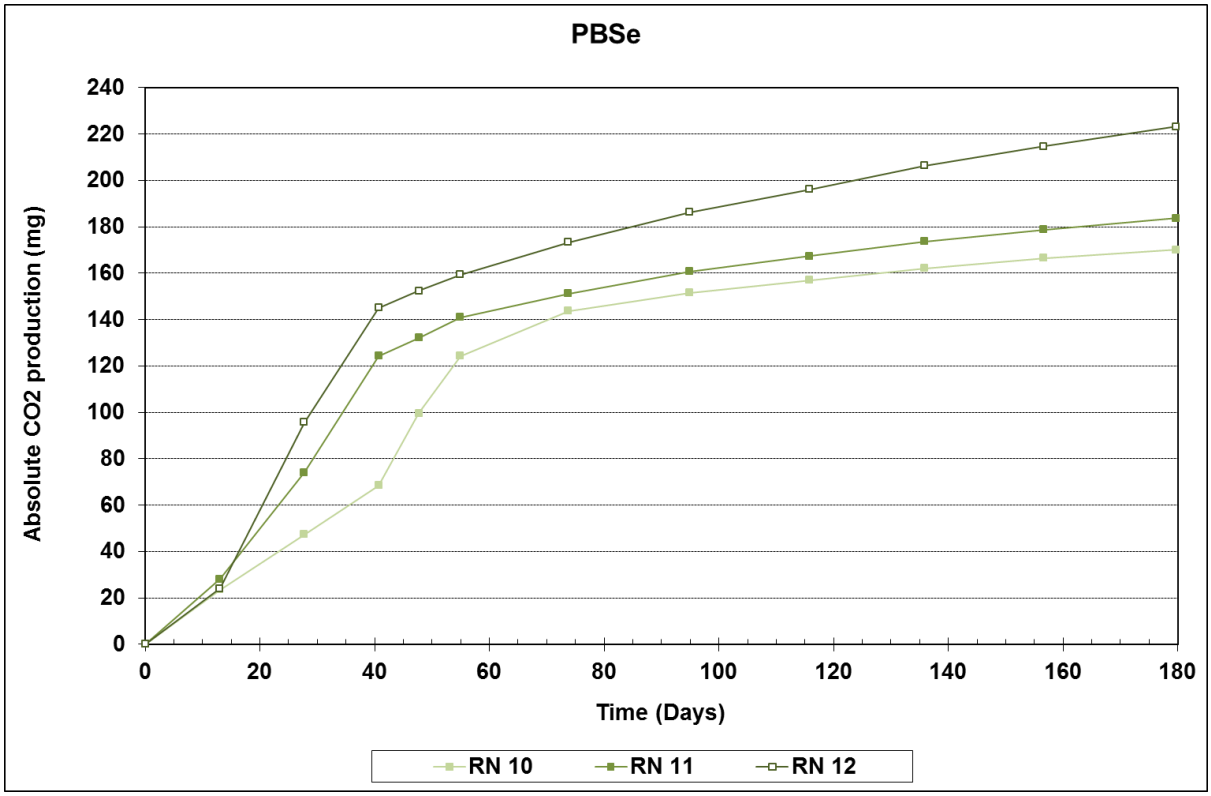


Figure 146. Total CO_2 production in PBSe reactors in seawater (Italy)

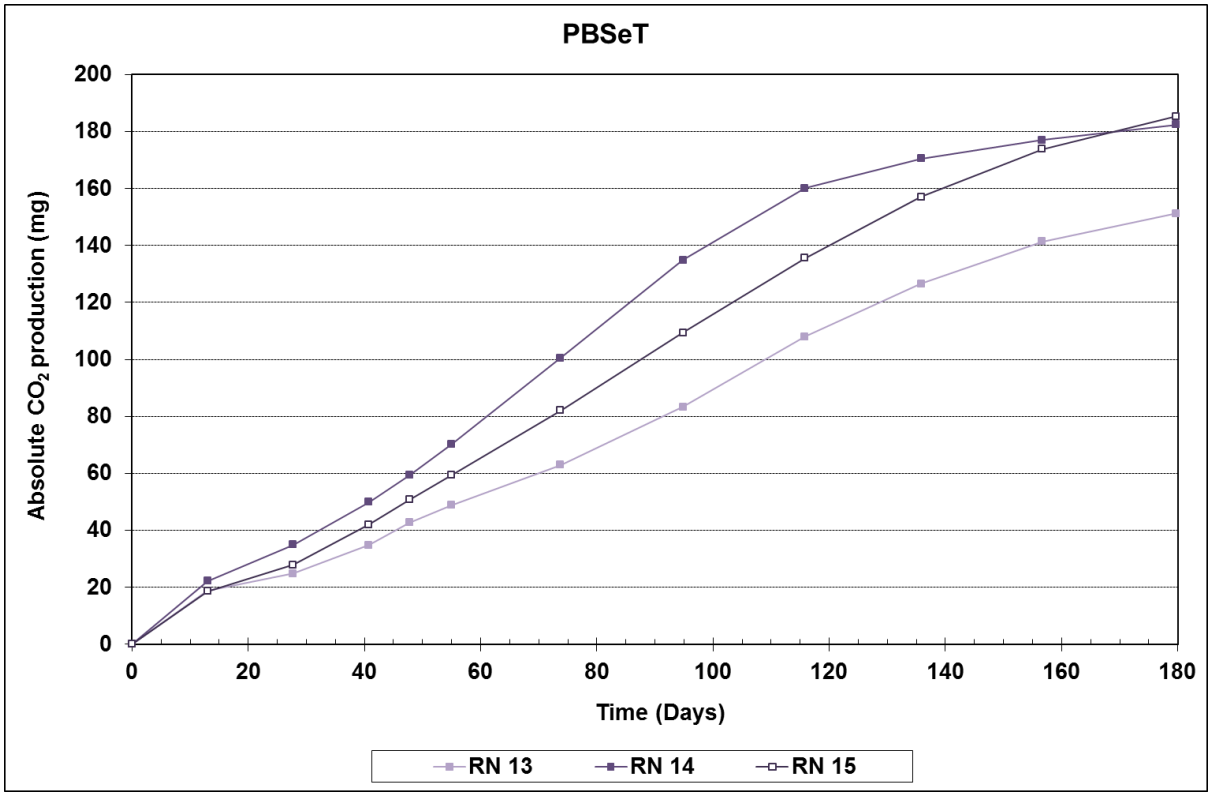


Figure 147. Total CO_2 production in PBSeT reactors in seawater (Italy)

Conclusions and recommendations

A summary of the biodegradation percentages is given in Figure 148.

Main observations:

- No or negligible biodegradation is measured for the negative reference material LDPE. This indicates that the test method is reliable.
- During the first year it was observed for positive reference material PHB copolymer that the biodegradation of 1 of the replicates (in both the Greek and the Italian seawater) remained increasing till unrealistic values. This indicates that it might be difficult to keep the system stable once a plateau has been reached. In spite of the fact that no modifications were implemented between the first year and the second year, this was not observed during the second year.
- PBSeT seems to be a difficult sample in seawater. Biodegradation is clearly slower when compared to PHB copolymer and PBSe, but significant differences can exist between replicates using the same seawater and between different types of seawater.

Recommendations:

- Performed testing was based on ASTM D6691 *Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Seawater Inoculum* (2009). In this test method a nitrogen source is added in a very high concentration (a factor 100 higher when compared to ISO 16221 and a factor 1000 higher when compared to OECD 306). It would be useful to revise the American standard test method (including a lower addition of nitrogen) or to prepare an European or International standard test method for the evaluation of the biodegradation of plastic materials in a pelagic environment including this more appropriate nitrogen level (e.g. 0.05 mg NH_4Cl/l). The very high nitrogen content of ASTM D6691 might cause problems due to nitrification in the systems.
- Variability between replicates might be caused by the low concentration of micro-organisms in natural seawater. Perhaps it could be an option to add a small amount of sediment to the seawater in order to increase the microbial activity (as sediment generally contains a higher concentration micro-organisms when compared to seawater). An alternative could be the addition of micro-organisms from a marine aquarium.

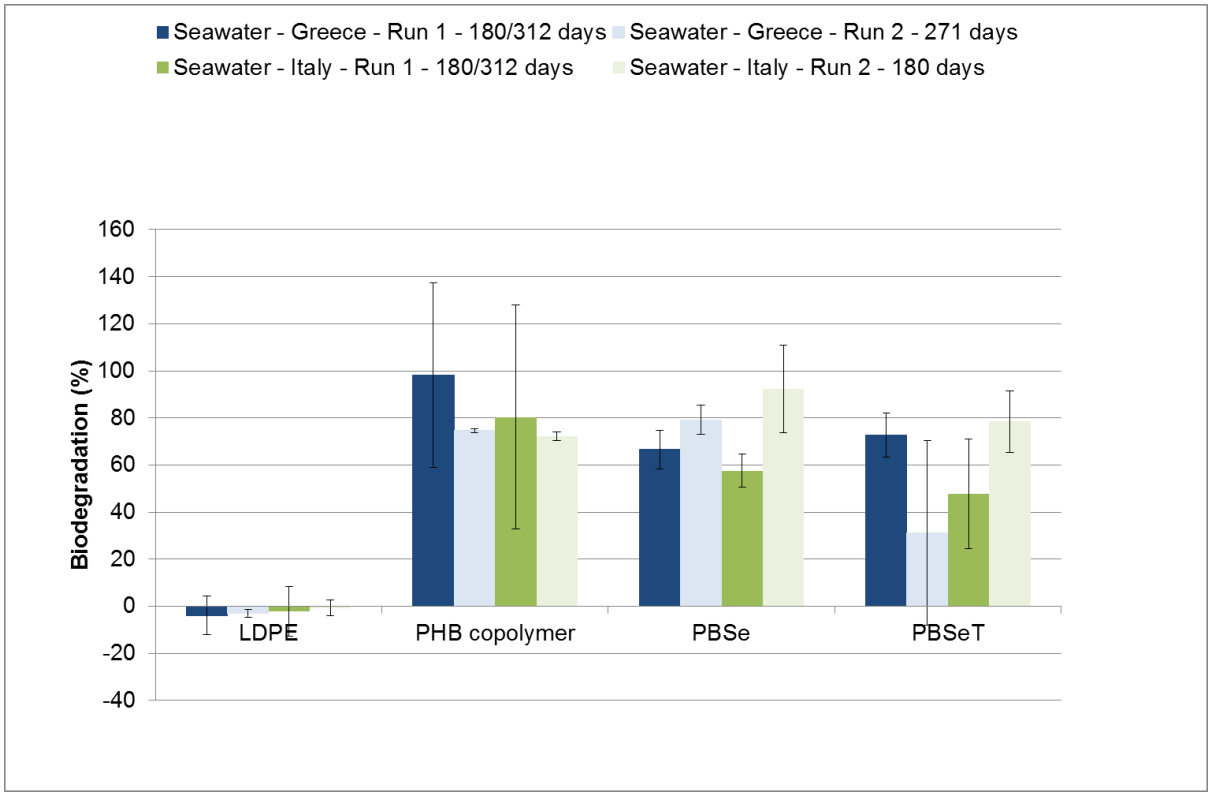


Figure 148. Overview biodegradation percentages at the end of the test

6.3 Laboratory results: LeAF

6.3.1 Biodegradation in Eulittoral zone

The results of the marine biodegradation tests with sandy marine sediment from Greece are given in Figure 149 (biodegradation) and Figure 150 (CO_2 production). The tests have been running for 224 days. The results with respect to the recovery of the test items after incubation are summarized in Table 55 and Figure 151. The results are a bit unexpected except for the negative control (LDPE) which is not degraded. Other than that T13 (with PBSeT) was producing a relatively large amount of CO_2 in the first week after addition of the test item, whereas the CO_2 production observed in the second week and thereafter was comparable to the other test bottles in this series. Furthermore, one PHB amended bottle (T8) is producing more CO_2 than the other PHB test bottles. Otherwise the CO_2 production observed in these second year tests is more or less similar in all test bottles, which suggest that all test items (except LDPE) are degraded at the same rate. This would normally indicate that something is limiting. It is difficult to determine which factor is rate limiting in this test set-up as is. The sediment was amended with $NaNO_3$ at the start of the incubation to avoid N-limitation. It is unclear whether this approach was successful since it is not possible to determine the N concentration after incubation.

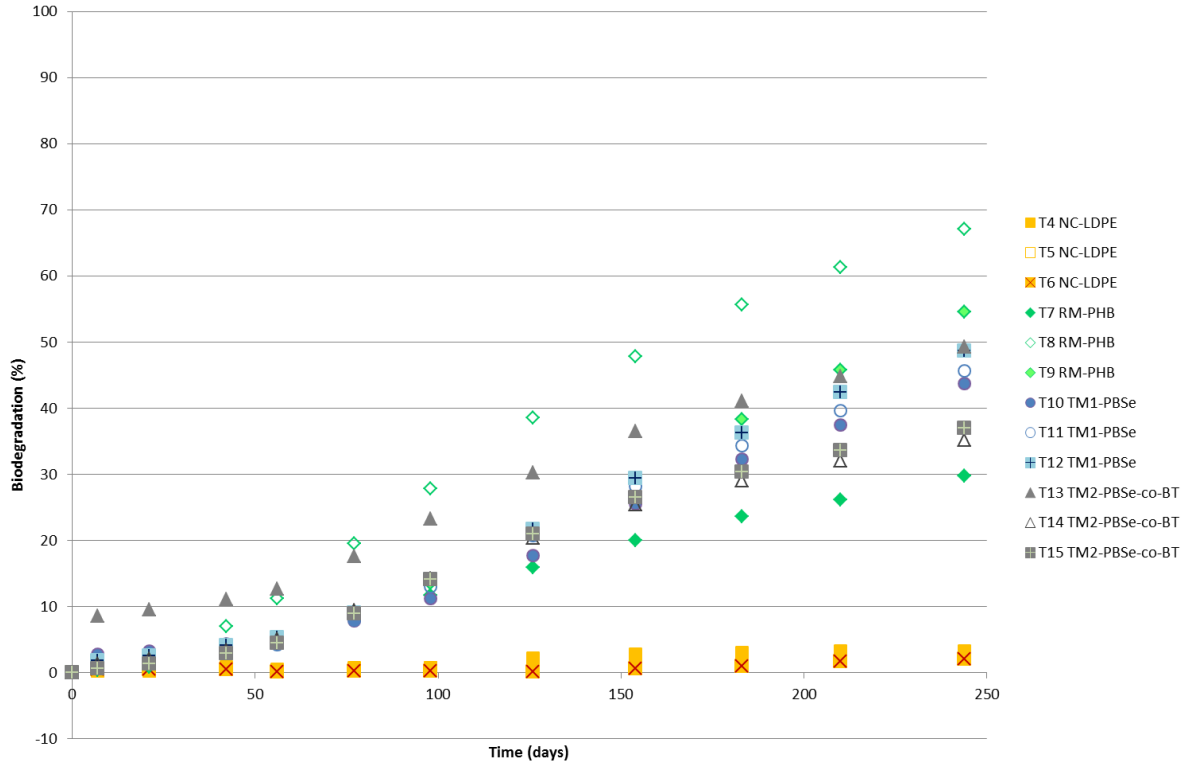
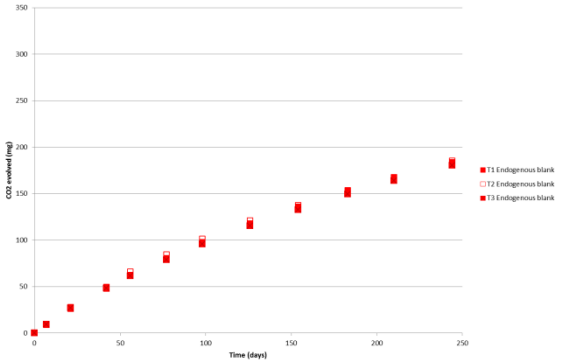
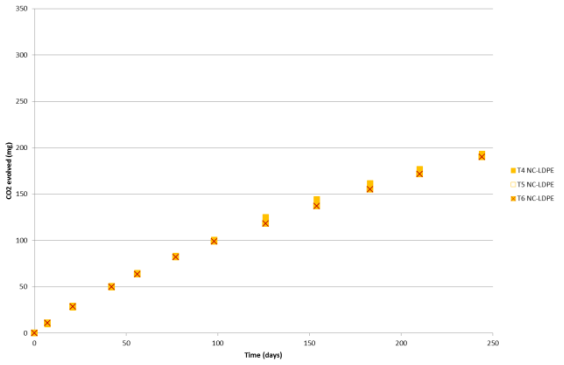


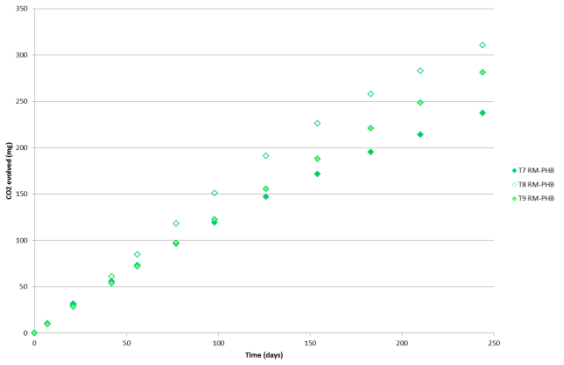
Figure 149. Biodegradation of test materials in sandy marine sediment from Greece.



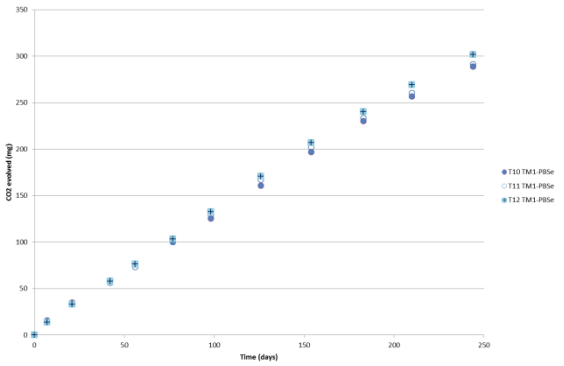
Endogenous blank



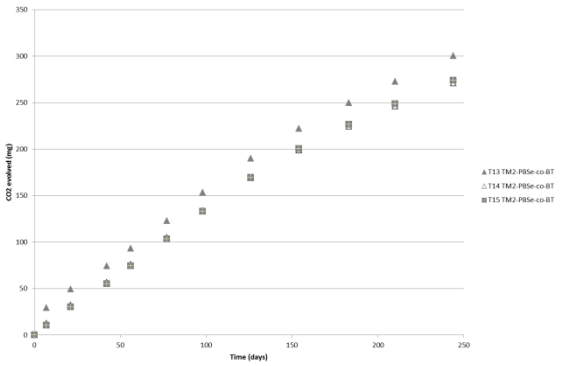
LDPE



PHB



PBSe



PBST

Figure 150. CO_2 production in the reactors with test materials and sandy marine sediment from Greece.

The VS (as % of TS) content of the sediments overall was lower at the end of the incubation, which indicates that a substantial amount of the VS in the sand was removed, but it is not clear how this occurred. On average it appears that 4.5 to 5.0 g of VS has disappeared during the incubation. This seems highly unlikely as this would amount to a CO_2 production of around 9 g/bottle.

The recovery percentages as compared to the %biodegradation observed showed differences between the different test items:

- LDPE: Biodegradation of the test items was not observed. From the CO_2 production it was calculated that the biodegradation was around 2.7%, which is negligible and substantiated by the recovery of LDPE which was between 103 and 107% of the initial weight. The sum of recovery and biodegradation is 105-110% which is in accordance with expectations. The retrieved test items were relatively clean but some organic material may have attached to the test items and as such may have been the reason for the somewhat higher weight recovered after incubation.
- PHB: The triplicate bottles showed a large deviation, which was backed up by the amount of PHB recovered after 224 days. Bottle T7 clearly lagged behind in CO_2 production and the piece of PHB in this bottle was largely intact. Bottles T8 and T9 were more active and the recovered PHB items much smaller. Overall the recovery of the test items is in accordance with the biodegradation (although clearly some organic material attached to the test items, which may have affected the T8 result).
- PBSe: The triplicate bottles showed similar results in a since that there was around 46% of biodegradation observed and little PBSe recovered. However the gap between test items (not)retrieved and biodegradation is substantial which suggests that intermediates of biodegradation accumulated in these tests
- PBSeT: Biodegradation was comparable to PBSe, but in all bottles around 70-80% of test item was retrieved. Also, in this case some organic material may have attached to the test items during incubation which may partially explain the high weight of the items after incubation.

Table 55. Results of the pH analysis and recovery of test items at the end of the test at day 224.

Bottle	pH end[a]	VS (%TS)[b]	Test item start (mg)	Test item end (mg)	Recovery (%)	Biodegradation (%)
T1 Endogenous blank	9.0	2.8				
T2 Endogenous blank	9.1	3.3				
T3 Endogenous blank	9.2	3.7				
T4 NC-LDPE	9.1	3.3	103	107	103	3.2
T5 NC-LDPE	9.0	3.0	100	103	103	2.9
T6 NC-LDPE	9.0	2.0	105	112	107	2.1
T7 RM-PHB	9.1	2.6	104	72	69	29.7
T8 RM-PHB	9.0	2.5	109	58	54	67.0
T9 RM-PHB	9.0	2.8	103	37	36	54.6
T10 TM1-PBSe	9.0	2.9	101	23	23	43.8
T11 TM1-PBSe	8.9	1.8	99	0	0	45.6
T12 TM1-PBSe	9.0	2.0	102	0	0	48.7
T13 TM2-PBSeT	9.0	1.9	100	70	70	49.2
T14 TM2-PBSeT	9.0	2.8	105	79	76	35.2
T15 TM2-PBSeT	9.0	2.3	103	79	77	37.0

[a] pH at the start in all bottles was pH 8.5; [b] VS at the beginning of the experiment was 3.8% of TS.

Example of completely retrieved LDPE test item

Retrieved PBSe test item in T10

Retrieved PHB test items from (from left to right) T7, T8 and T9

Retrieved PBSeT test items from (from left to right) T13, T14 and T15

Figure 151. The test items recovered after 224 days of incubation

Concluding remarks

The tests were not very difficult to set up but in the end several issues came up with regards to set up and interpretation of the data:

- It is unclear whether aerobic conditions prevail in the sand when the test item is covered with a layer of sand (as is prescribed in the test set-up). A thinner layer would perhaps be more realistic;
- It is unclear whether the addition of nutrients had a beneficial effect on the test outcome. In fact the lag phase in the 2nd year of testing (with nitrate dosage) was comparable to the lag phase in year one. The differences amongst triplicates comparable.
- The difference in CO_2 production between endogenous blank and positive control were relatively small (CO_2-PHB/CO_2-endogenous <1.8 in both years). A larger amount of test item, i.e. more CO_2 produced in test as compared to endogenous blank) may improve the results.

6.3.2 Biodegradation in Benthic zone

The results of the marine biodegradation tests at the sediment/seawater interface from Greece are given in Figure 152 (biodegradation) and Figure 153 (CO_2 production). The tests have been running for 233 days. The results with respect to the recovery of the test items after incubation are summarized in Table 56 and Figure 155. Similar to the tests with the sandy marine sediment described in section 6.3.1, the results are deviating amongst triplicates. Furthermore, there is a nett biodegradation observed in one of the negative control (LDPE) bottles (BS4) which is unexpected. This however, may also be related to the relatively small amount of test sample used in the test and the relative large CO_2 production in the endogenous control. These factors together with the (small) differences amongst the LDPE triplicate bottles may cause the nett large effect in the biodegradation (Figure 152). Also, in this test the biodegradation is really taking off ahead of the other bottles in one of the PHB triplicate bottles (BS9). The results of the PBse and PBSeT incubations are more or less along the line of expectations. Similar to last year black staining was observed in the most active bottle (BS9). The black stains were visible both at the top and the bottom of the sediment layer (Figure 154). Again, and similar to tests in the previous year, this may be an indication for the occurrence of anaerobic events during incubation. Since the Erlenmeyers were not shaken this may be due to limited oxygen diffusion. In the course of the experiment the black stains disappeared.

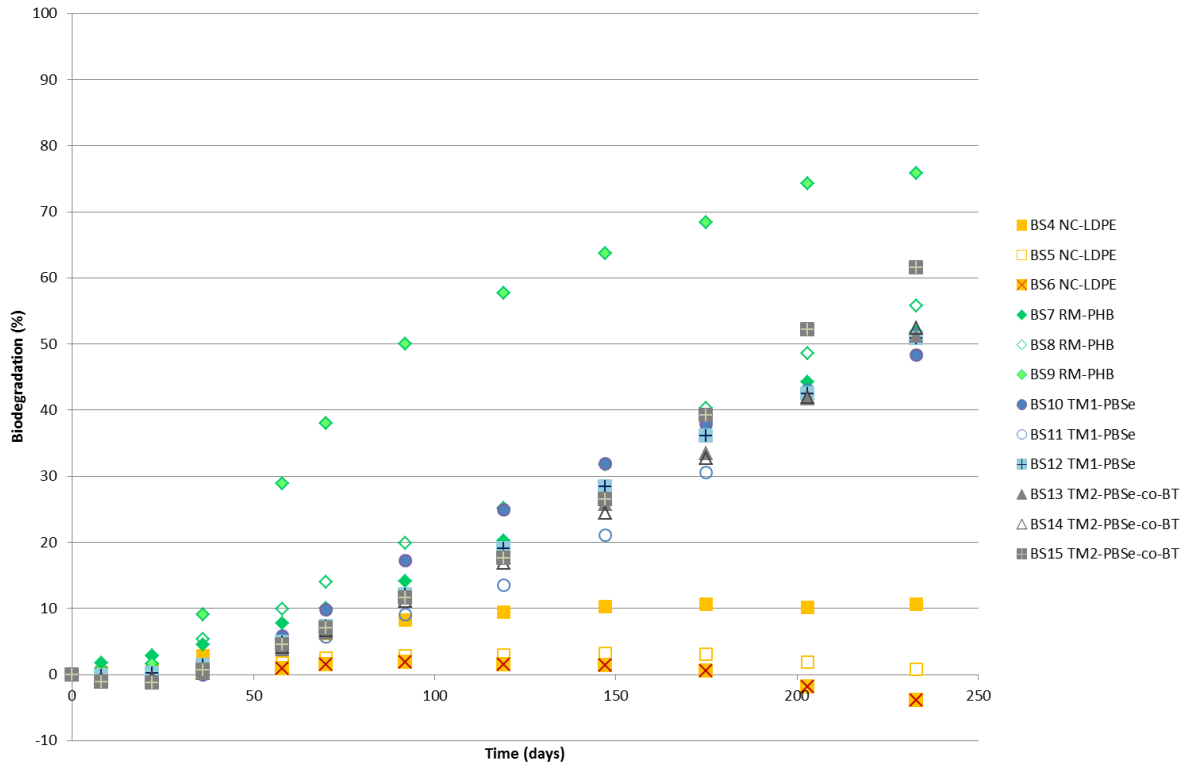
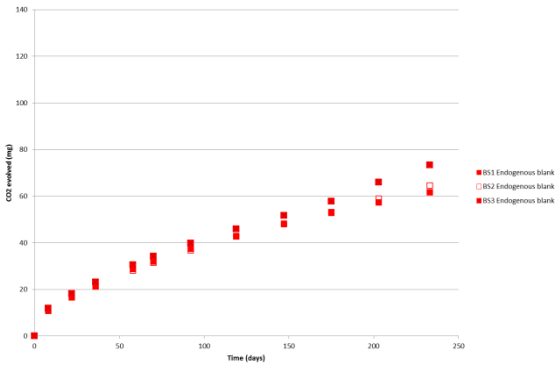
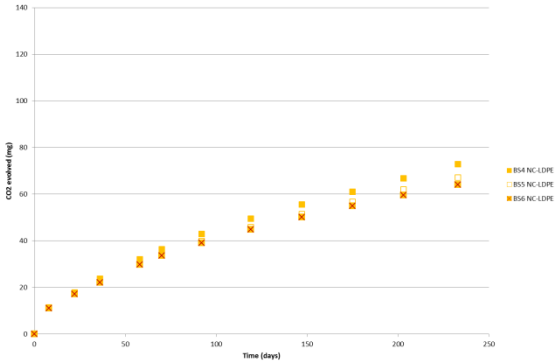


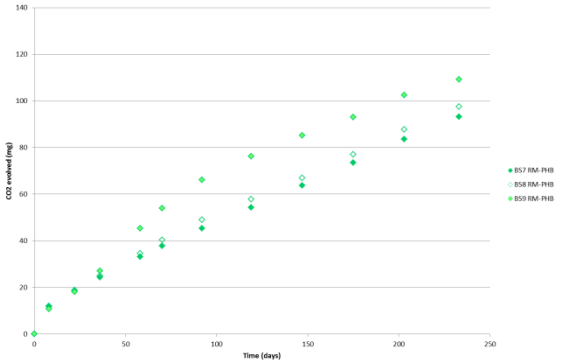
Figure 152. Biodegradation of test materials at sediment/seawater interface from Greece.



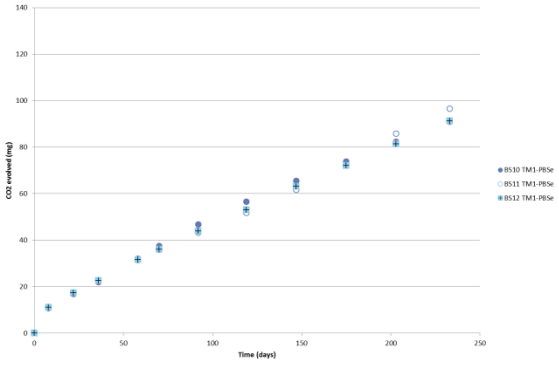
Endogenous blank



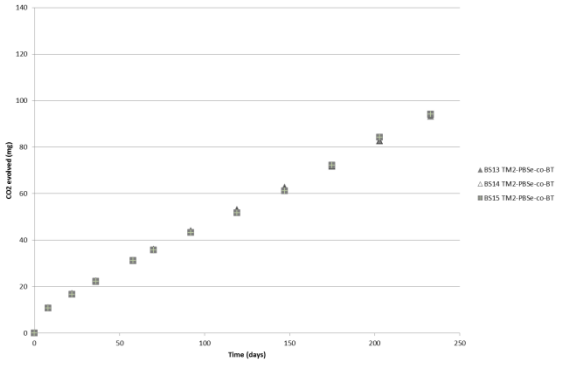
LDPE



PHB



PBSe



PBSe-co-BT

Figure 153. CO_2 production in the reactors with test materials at sediment/seawater interface from Greece.

Figure 154. Black staining in bottle BS9 with Greek sediment/seawater and PHB.

Table 56. Results of the pH analysis and recovery of test items at the end of the test at day 233.

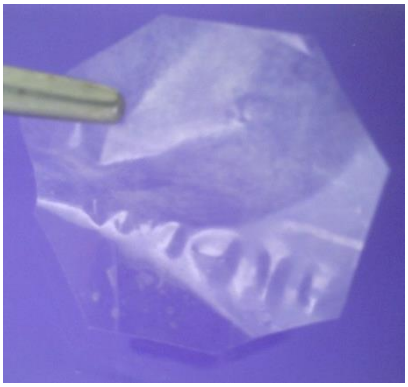
Bottle	pH start	pH end	VS (%TS)[a]	Test item start (mg)	Test item end (mg)	Recovery (%)	Biodegradation (%)
BS1 End. blank	7.9	8.3	7.40	-	-	-	-
BS2 End. blank	7.9	8.3	7.25	-	-	-	-
BS3 End. blank	7.9	8.3	5.93	-	-	-	-
BS4 NC-LDPE	7.9	8.3	5.93	19.31	20.80	108	10.7
BS5 NC-LDPE	7.9	8.4	6.68	21.61	23.60	109	0.8
BS6 NC-LDPE	8.0	8.3	6.16	20.62	21.90	106	-3.9
BS7 RM-PHB	8.0	8.3	7.05	29.46	16.90	57	51.8
BS8 RM-PHB	8.0	8.3	6.93	31.74	13.10	41	55.7
BS9 RM-PHB	8.0	8.3	9.25	32.10	0.00	0	75.8
BS10 TM1-PBSe	8.0	8.3	8.87	21.16	18.00	85	48.3
BS16 TM1-PBSe	8.0	8.3	8.41	24.34	15.80	65	51.5
BS12 TM1-PBSe	8.0	8.3	7.95	20.27	15.30	75	50.9
BS13 TM2-PBSeT	7.9	8.2	7.59	21.86	12.10	55	51.2
BS14 TM2-PBSeT	8.0	8.3	8.45	21.85	13.50	62	52.4
BS15 TM2-PBSeT	7.8	8.3	7.05	18.80	10.80	57	61.6

[a] VS at the beginning of the experiment was 5.22% of TS.

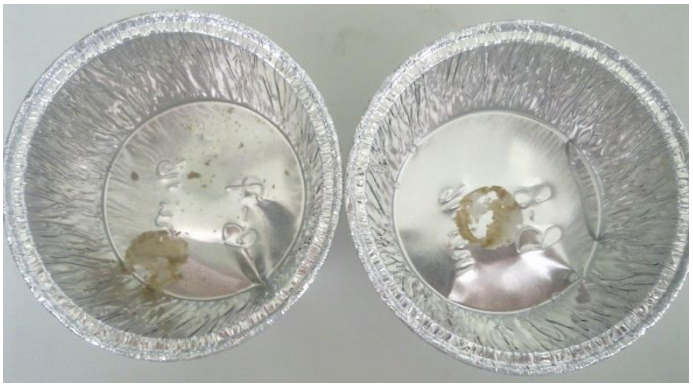
The VS (as % of TS) content of the sediments overall was higher at the end of the incubation, which indicates that a substantial amount of the VS was formed. On average it appears that 0.3 gram of VS was formed in addition to the 1.6 gram present at the start of the incubation. The recovery percentages as compared to the %biodegradation observed showed differences between the different test items. Overall the sum of the amount of recovered test item and the biodegradation is around 110% or higher. This may have been caused by inaccurate biodegradation results (overestimation of biodegradation in the CO_2 analysis) and/or dirt attached to the test items. The latter was in most cases at least partially

responsible for the high recoveries. Most recovered test items were covered with a brown matter which could not be removed.

- LDPE: Except for the apparent biodegradation observed in bottle BS4, there was little or no CO_2 production. The recovery of LDPE which was between 106 and 109% of the initial weight. The sum of recovery and biodegradation is 100-120% which is somewhat higher than expected. The retrieved test items were relatively clean but some organic material may have attached to the test items.
- PHB: The triplicate bottles showed a large deviation, which was backed up by the amount of PHB recovered after 233 days. The sum of test items recovered and biodegradation was 75 to 108%. Bottle BS9 with no recoverable test item showed the highest biodegradation percentage. The PHB in the other test bottles was clearly affected and the part removed was largely recovered as CO_2. In this case all the balance is rather nice although the test items are brown.
- PBSe: The sum of recovered test item and biodegradation is too high in the three bottles. Results were similar for the triplicate bottles
- PBSeT: Biodegradation of was comparable to PBSe, but in all bottles around 55-62% of test item was retrieved.

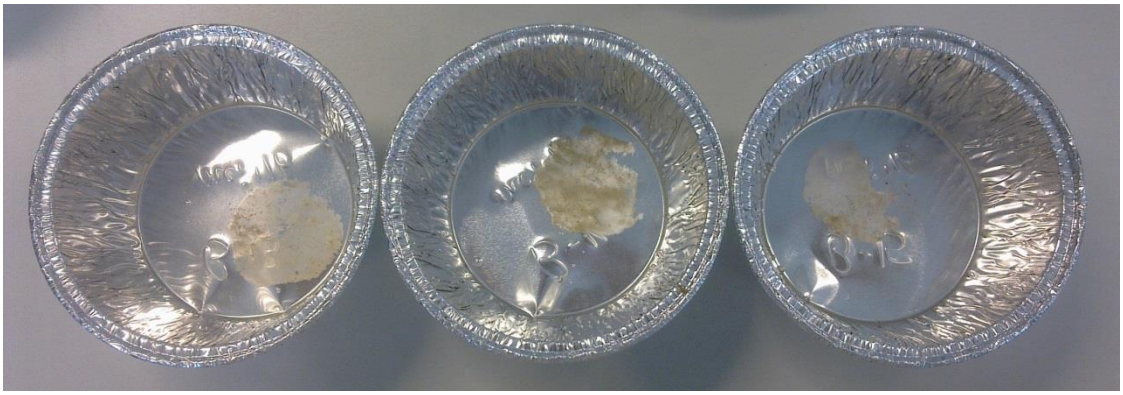


Example of completely retrieved LDPE test item



Retrieved PHB test items from (from left to right) BS7, and BS8

Retrieved PBSeT test items from (from left to right) BS10, BS11 and BS12



Retrieved PBSeT test items from (from left to right) BS13, BS14 and BS15

Figure 155. The test items recovered after 233 days of incubation

Concluding remarks

Two different test item materials (PBSe and PBSeT) and a positive control (PHB) have been used in biodegradation tests for two different marine environments: sandy sediment environment and the interface between sediment and seawater (benthic). Two tests were carried out at two consecutive years. The first year with material from the Elba test site and the second year with material from the Greek test site (Salamina). Each year a negative control (LDPE) was included in the tests.

The tests were not very difficult to set up but in the end several issues came up with regards to set up and interpretation of the data:

- Anaerobic conditions were prevailing underneath the sample and at the bottom of test bottles during a certain period of the test period in both test years as evidenced by the appearance of black spots in the test flasks directly underneath the PHB test items. This black stain formation is probably related to the formation of sulphide precipitates. This suggests that there is a lack of oxygen around the more rapidly degraded test items. It is therefore uncertain whether the % of biodegradation is accurately calculated in the case of PHB and other test items as well (since the black stains may only be visible when oxygen depletion is at its worst) since the biodegradation may also at least partially be carried out by anaerobic (sulphate

reducing) bacteria. This staining did not occur with the negative control. Mild shaking to induce oxygenation of the top layer of the sediment may be beneficial to sustain aerobic conditions throughout the test period.

- Amount of sample: the amount of test item may be increased to decrease test duration time and to increase the differences between CO_2 evolved from the endogenous blanks and the amount of CO_2 produced in the active test bottles.
- Nutrients were not added in the test. It is unclear whether the addition of nutrients would have been beneficial for the test outcome.

When comparing the results from the different test sites and the different years (Table 57) it is apparent that:

- LDPE was behaving similarly in all conditions (with the possible exception of the "Greek benthic test");
- PHB was suitable as a positive control, although the results obtained in the 2nd year showed rather high deviations;
- The test items showed different results for each environment and for the different years.

It has to be noted that the tests in the 2nd year (with the Greek sediment and seawater) have run shorter than in year 1.

Table 57. Overview of results obtained for the negative and positive control and the test items with sediments of the different locations in two consecutive years.

	Sandy sediment Elba	Sediment-seawater interface Benthic Elba	Sandy sediment Elba Greek	Sediment-seawater interface Benthic Greek
Recovery (%)				
LDPE	99.7±5.9	106±3.1	104.3±2.3	107.7±1.5
PHB	0.0±0.0	0.0±0.0	53.0±16.5	32.7±29.4
PBSe	11.7±18.5	0.0±0.0	7.7±13.3	75.0±10.0
PBSeT	8.3±14.4	4±7.5	74.3±3.8	58.0±3.6
Biodegradation (%)				
LDPE	-1.0±1.8	-2.0±1.4	2.7±0.6	2.5±7.5
PHB	71.3±10.7	82.4±6.9	50.4±19.0	61.1±12.9
PBSe	37.9±5.8	76.1±2.3	46.0±2.5	50.2±1.7
PBSeT	36.3±0.8	71.0±4.3	40.5±7.6	55.1±5.7

6.4 Laboratory results: BASF

Table 58. Biodegradation results obtained in the three environment studied used the Italian inoculum

Italian inoculum	days	Sample	Biodegradation (%)
Eulittoral	50	Blank	/
		LDPE	-1.6 ± 1.4
		PBSe	8.8 ± 1.3
		PBSeT	4.2 ± 3.9
		PHB	43.3 ± 4.4
		Filter paper	19.4 ± 2.9
	100	Blank	/
		LDPE	-3.4 ± 1.0
		PBSe	26.8 ± 3.1
		PBSeT	15.4 ± 4.4
		PHB	57.0 ± 5.4
		Filter paper	25.2 ± 3.8
	150	Blank	/
		LDPE	-2.3 (only 1 replicate available)
		PBSe	50.2 ± 0.9
		PBSeT	31.5 ± 4.8
		PHB	68.1 ± 6.8
		Filter paper	32.9 ± 3.8
	210	Blank	/
		LDPE	-0.5 (only 1 replicate available)
		PBSe	64.8 ± 3.0
		PBSeT	44.2 ± 4.9
		PHB	75.5 ± 5.9
		Filter paper	40.8 ± 3.8
Benthic	50	Blank	/
		LDPE	-0.2 ± 1.2
		PBSe	7.3 ± 1.6
		PBSeT	8.3 ± 3.5
		PHB	19.2 ± 8.1
		Filter paper	8.0 ± 0.8
	100	Blank	/
		LDPE	-1.6 ± 1.1
		PBSe	30.0 ± 7.9
		PBSeT	31.7 ± 4.5

		PHB	38.4 ± 13.0
		Filter paper	23.2 ± 2.3
	150	**Blank**	/
		LDPE	-2.6 (only 1 replicate available)
		PBSe	53.3 ± 7.1
		PBSeT	54.5 ± 6.7
		PHB	50.5 ± 10.9
		Filter paper	42.1 ± 4.3
	220	**Blank**	/
		LDPE	-1.1 (only 1 replicate available)
		PBSe	74.3 ± 2.8
		PBSeT	68.7 ± 1.1
		PHB	69.2 ± 1.5
		Filter paper	51.0 ± 1.6
Pelagic	**50**	**Blank**	/
		LDPE	3.5 ± 2.5
		PBSe	14.9 ± 3.4
		PBSeT	7.4 ± 0.8
		PHB	83.5 ± 2.8
		Filter paper	13.6 ± 7.5
	100	**Blank**	/
		LDPE	7.5 ± 0.0
		PBSe	37.6 ± 11.8
		PBSeT	7.6 ± 6.0
		PHB	86.9 ± 1.2
		Filter paper	27.5 ± 8.3
	150	**Blank**	/
		LDPE	6.1 ± 2.5
		PBSe	61.7 ± 16.1
		PBSeT	15.3 ± 14.0
		PHB	84.3 ± 1.2
		Filter paper	35.3 ± 2.1
	220	**Blank**	/
		LDPE	/
		PBSe	78.6 ± 7.5
		PBSeT	44.8 ± 8.7
		PHB	/
		Filter paper	40.4 ± 1.3

6.5 Laboratory results: AUA

6.5.1 Biodegradation in Eulittoral zone

Sandy sediment was collected from the eulittoral zone of the shoreline in Greece (Salamis area), where the sediment is submerged in seawater at times due to waves. The sediment was filtered so as to remove coarse organic or inorganic articles in order to obtain a homogeneous sandy substrate. The properties of the sediment used for the eulittoral lab tests year 2, were determined at the lab of Soil Science and Agricultural Chemistry of AUA and are presented in Table 59.

Table 59. Properties of sediment used for the tidal test of 2nd year

Properties of sediment	
Org C %	0.058-0.078
Total N %	0.021-0.024
Mechanical composition	
C %	2.38-3.56
Si %	2-2.18
S %	94.44-95.44

The sediment was transported and stored at approximately 4°C. The experimental procedure for the eulittoral test is similar of this of 1st year. Approximately 356 g of sediment is placed in the bottom of each vessel. Glass vessels of approximately 4L volume that can be sealed air-tight are used. The sediment is enriched with nitrogen (by adding the appropriate weight of fertilizer to a C: N ratio equal to 10:1 (w/w)). The same amount of nutrients was also added in the blanks. The weight of all vessels is recorded and the samples of the test materials are added (approximately 1000 mg organic carbon). The samples are placed in the vessels in the following way: about 100 g of sediment is removed from the layer in the bottom of the reactor. This sediment is kept in a clean container. The test specimen is laid down on top of the remaining sediment. No specimen is placed in the blank reactors. The withdrawn sediment is put back in the reactor to form a homogenous layer that covers the specimens. The experiment included 2 blanks, 2 PHB, 2 PBSe of 25 μm thickness, 2 PBSeT of 25 μm thickness, 2 PBSe of 100 μm thickness, 2 PBSeT of 100 μm thickness and 2 PE replicates. The initial weight of the reactors is recorded. The CO_2 absorbing solution and water are introduced in beakers containing 50 ml KOH 0.2N for the blanks and the PE samples and 0,5N for all other samples and 50ml distilled water and the vessels are sealed and placed in a darkened chamber or cabinet, where the temperature is maintained between 25 ± 2 °C. The experiment did not include a preliminary phase. The amount of CO_2 produced is determined by titrating the remaining potassium hydroxide with 0.25 N hydrochloric acid. The container for the CO_2 absorber is removed and titrated twice per week for the first 2 to 3 weeks and every 1 to 2 weeks thereafter. When the CO_2 absorbers are removed, the reactors are allowed to stay open so that the air is refreshed before replacing 50 ml of fresh potassium hydroxide and resealing the reactor. The reactors remain open for approximately

10 min. The experimental set up of the test is shown in Figure 156. The parameters of the test are presented in Table 60.

Figure 156. Experimental set up for the eulittoral test (2nd year)

Table 60. Parameters of the eulittoral test of 2nd year

Tidal test **Parameters**	
Reactor volume (L)	4 .0
Type	Static (closed vessel)
Temperature (°C)	25-28
Sample characteristics	Piece of plastic film
Quantity of sample (mg)	1500 mg – 2000 mg of sample corresponding to 1000 mg of organic carbon
Quantity of inoculum (g)	Around 350 (after sieving)
Measurement method	CO_2 titration
Nutrients: N(g)/sample TOC (g)	0.1/1.0

The weights of the materials and the corresponding organic carbon content are summarized in Table 61.

Table 61. Weights and corresponding organic carbon of materials used in the eulittoral test (2nd year)

Thickness (µm)	Material	Weight of sample (mg)	Organic carbon in sample (mg)
80	PHB 1	2095.2	1001.9
80	PHB 2	2091.0	999.9
25	PBSe 1	1576.0	1028.5
25	PBSe 2	1581.9	1032.3
25	PBSeT 1	1549.2	1010.9
25	PBSeT 2	1543.1	1006.9
100	PBSe 1	1538.9	1004.3
100	PBSe 2	1533.5	1000.8
100	PBSeT 1	1534.2	1001.1
100	PBSeT 2	1532.1	999.7
20	LDPE 1	1170.1	994.9
20	LDPE 2	1157.8	984.5

The evolved over theoretical CO_2 production was monitored for a period of 134 days and the analytical results are presented in Figure 157, Figure 158 and Figure 159 and in Table 62.

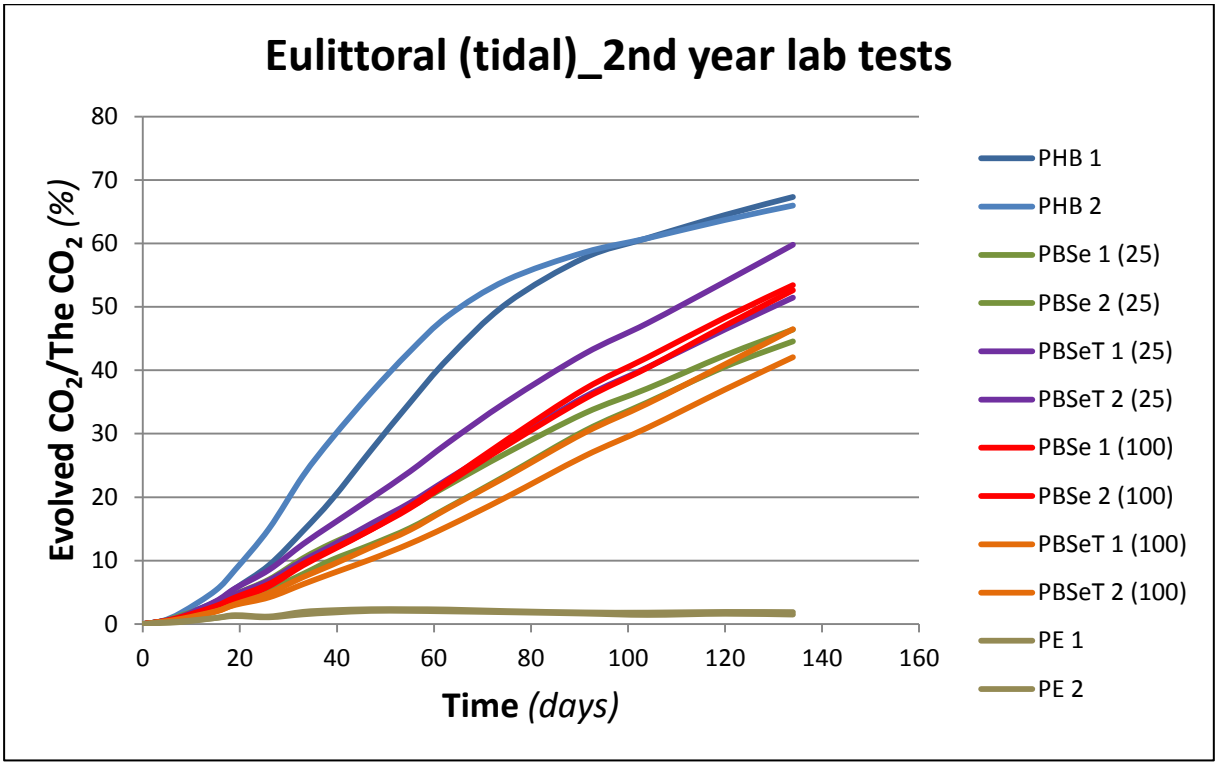


Figure 157. Evolved over theoretical CO_2 % vs. time for the eulittoral test of 2nd year

Table 62. Evolved over theoretical CO_2 production (average and standard deviation) vs. time for the eulittoral test of 2nd year

Days	PHB	PBSe (25)	PBSeT (25)	PBSe (100)	PBSeT (100)	LDPE
63	45.3±4.7	20.2±2.5	25.8±4.1	22.5±0.3	16.9±2.0	2.1±0.2
75	52.4±2.6	25.2±2.5	31.8±4.6	28.6±0.6	21.6±2.3	1.9±0.1
91	58.1±0.6	31.8±1.9	39.2±4.8	36.2±1.1	28.3±2.6	1.7±0.1
120	64.1±0.5	41.4±1.3	50.2±5.3	47.7±0.9	39.0±2.8	1.8±0.2
134	66.6±0.9	45.5±1.3	55.6±5.9	53.0±0.6	44.3±3.1	1.7±0.3

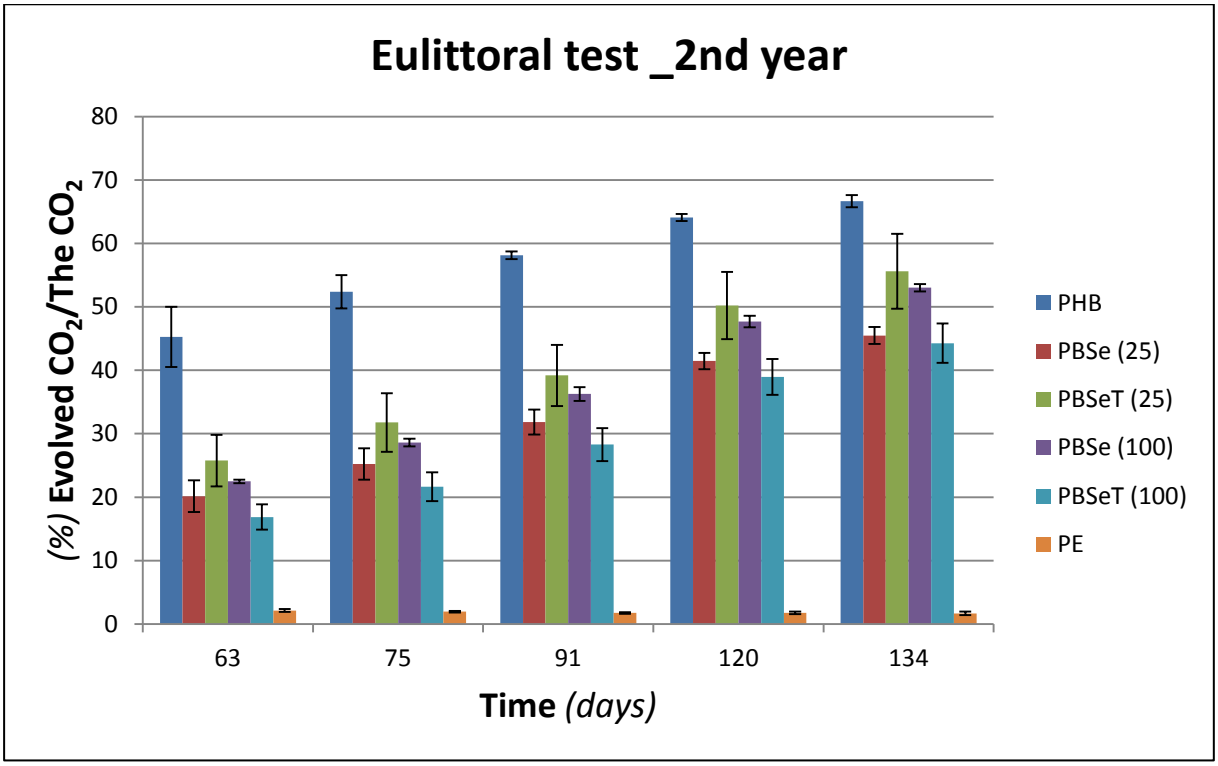


Figure 158. Average evolved over theoretical CO_2 % (average and standard deviation) vs. time for the eulittoral test of 2nd year

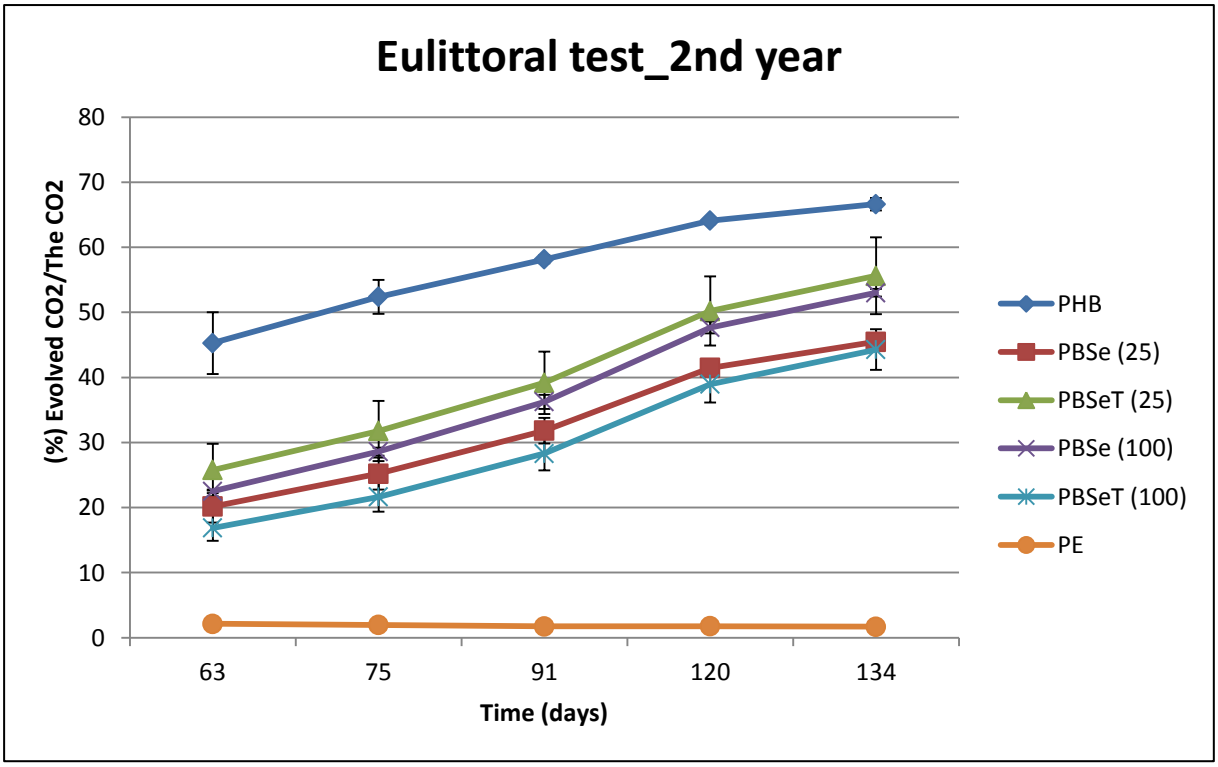


Figure 159. Average evolved over theoretical CO_2 % (average and standard deviation) vs. time for the eulittoral test of 2nd year

As it can be concluded from the results the average evolved over theoretical CO_2 % follows the order PHB> PBSeT (25)> PBSe(100)> PBSe(25)> PBSeT(100) >LDPE. After 134 days the average evolved over theoretical CO_2% is 66.6% ± 0.9% for the PHB, 45.5% ± 1.3% for the PBSe (25), 55.6% ± 5.9% for the PBSeT (25), 53.0% ± 0.6% for the PBSe (100), 44.3% ± 3.1% for the PBSeT (100) and 1.7% ± 0.3% for the LDPE. These results do not clearly show that the thickness influences the biodegradation rate. The standard deviation values are relatively low and consequently the results can be considered reliable.

6.5.2 Biodegradation in Benthic zone

Experimental procedure

Seawater and sediment from the sea bottom were sampled separately near the shoreline in Salamis island area in Greece at 18-20 m depth. The properties of the sediment used for the sublittoral lab tests were determined at the Soil Science and Agricultural Chemistry laboratory of AUA and are presented in Table 63.

Table 63. Properties of sediment used for the sublittoral tests of 2nd year

Properties of sediment	
Org C %	0.312
Total N %	0.035
Mechanical composition	
C %	3.56
Si %	7.18
S %	89.26

The sediment and seawater were transported and stored at approximately 4°C. The experimental procedure for the benthic sublittoral tests is similar of the one of the 1st year: 170 g of wet sediment are placed on the bottom of each reactor. Then 380 ml of natural seawater is added. The carbon dioxide absorber consists of 50 ml of KOH 1N. The flasks are kept at constant-temperature 25°C and the CO_2 evolution is monitored. The plastic film samples are placed on the sediment-water interphases of the reactors and are covered by a thin layer of sediment in order to keep them underwater. The sample quantity depends on the organic content of the material so as to correspond to the same selected amount of organic carbon. The mass of the sediment, the mass of the specimen and the volume of seawater added, for each vessel is recorded. This experiment did not include a preliminary phase. The experimental set up for the sublittoral test of 2nd year is presented in Figure 160. The amount of CO_2 produced is determined by titrating the remaining potassium hydroxide with 0.25 N hydrochloric acid by automatic titrator. The CO_2 absorbers are titrated every 4 days for the first 2 to 3 weeks and every 1 to 3 weeks thereafter. At the time of removal of the

containers, the reactor is allowed to stay open so that the air is refreshed before replacing 50 ml of fresh potassium hydroxide and resealing the reactor. The reactors remain open approximately for 15 min.

1st series of benthic lab tests

The first series of experiments for the sublittoral environment were performed by using 300 mg of organic carbon for each material sample. That means approximately 627 mg sample for the PHB materials (47.82% organic carbon content) and approximately 460 mg sample for the PBSe and PBSeT materials (organic carbon content 65.26% and 65.25% respectively). The exact weights and corresponding organic carbon of the samples are presented in Table 64 showing the test set-up. The experiment included 3 blanks, 3 PHB, 3 PBSe of 25 μm thickness, 3 PBSeT of 25 μm thickness, 3 PBSe of 100 μm thickness, 3 PBSeT of 100 μm thickness, 1 LDPE and 2 PLA (27 μm thickness) replicates. The fertilizer used to enrich the sediment in nutrients had composition N-P-K: 13-2-44. It includes nitrogen in nitric form. The weight of the additional fertilizer used was calculated based on a ratio of N/C equal to 1/10 with respect to the organic carbon contained in the sample. The appropriate amount of fertilizer was dissolved in natural seawater and added to each reactor. As mentioned above 170 g of sediment were added to each reactor and the volume of natural seawater plus the natural seawater with the fertilizer added in each reactor was approximately 400 ml. The CO_2 production determined by titration was monitored for a period of 201 days. The parameters of the sublittoral lab test of 2nd year (1st series) are presented in Table 65. The analytical results of the evolved over theoretical CO_2 are presented in Figure 161, Figure 162 and Figure 163 and in Table 66.

Figure 160. Experimental setup of the sublittoral test of 2nd year

Table 64. Weights and corresponding organic carbon of samples used for the sublittoral test of 2nd year

Nominal Thickness (µm)	Material	Weight of sample (mg)	Organic carbon in sample (mg)
80	PHB 1	628.4	300
80	PHB 2	627.4	300
80	PHB 3	627.3	299
25	PBSe 1	466.0	304
25	PBSe 2	467.7	305
25	PBSe 3	468.6	305
25	PBSeT 1	455.0	296
25	PBSeT 2	437.4	285
25	PBSeT 3	457.7	298
100	PBSe 1	456.7	298
100	PBSe 2	457.8	298
100	PBSe 3	449.5	293
100	PBSeT 1	461.7	301
100	PBSeT 2	470.9	307
100	PBSeT 3	463.7	302
27	PE	367.2	312
27	PLA 1	602.5	301
27	PLA 2	606.8	303

Table 65. Parameters of the sublittoral lab test of 2nd year (1st series)

Sublittoral test Parameters	
Reactor volume (L)	4 .0
Type	Static (closed vessel)
Temperature (°C)	25-28
Sample characteristics	Piece of plastic film
Quantity of sample (mg of organic carbon)	300
Quantity of inoculum (g)	Around 170
Measurement method	CO_2 titration
Chemical reagent	KOH 1N, 0.5 N, 0.2 N

	and HCl 0.25M
Seawater (ml)	Natural (around 400)
Nutrients: N (g)/sample TOC (g)	0.1/1.0

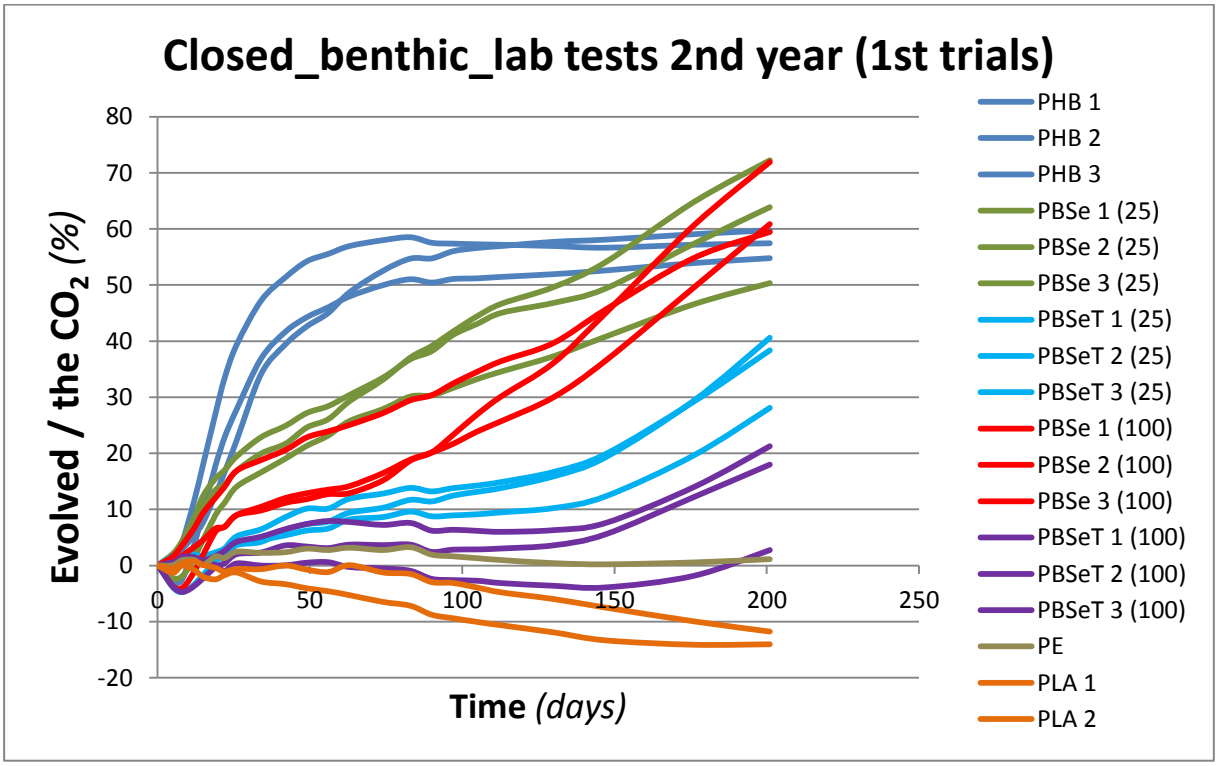


Figure 161. Evolved CO_2/the CO_2 % vs. time for the sublittoral lab test of 2nd year (1st series)

Table 66. Evolved CO_2/theoretical CO_2 % vs. time for the sublittoral lab test (1st series)

Days	PHB	PBSe (25)	PBSeT (25)	PBSe (100)	PBSeT (100)	PE	PLA
63	51.2±5.0	28.5±2.4	9.8±1.9	17.4±6.7	2.2±4.0	3.2±0.0	-2.6±3.8
97	54.8±3.3	38.1±5.6	11.7±2.5	25.8±5.8	2.2±4.5	1.6±0.0	-6.3±4.4
130	55.5±3.1	44.6±6.5	14.3±3.5	35.2±4.9	2.1±5.1	0.4±0.0	-8.9±4.2
176	56.7±2.6	56.2±9.2	26.1±5.6	55.0±5.5	8.1±8.7	0.5±0.0	-12.0±2.9
201	57.3±2.5	62.1±11.0	35.7±6.7	64.1±6.8	14.0±9.9	1.1±0.0	-12.9±1.6

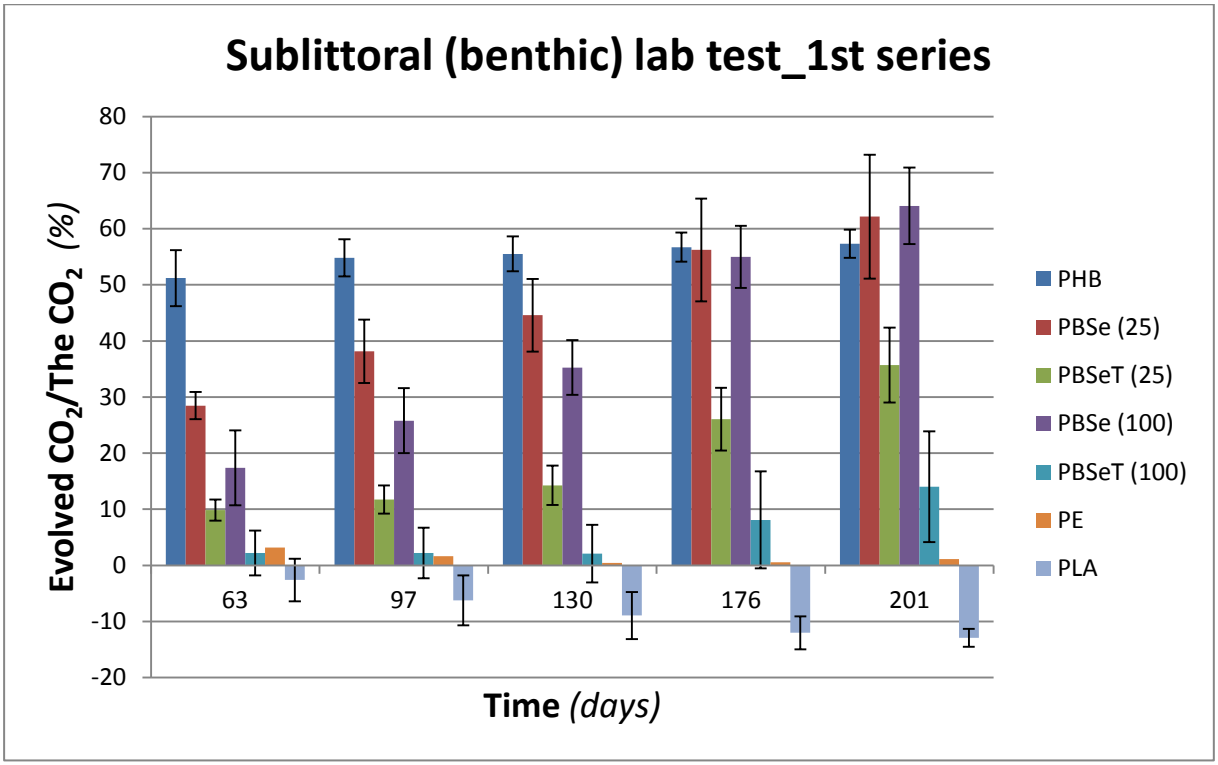


Figure 162. Evolved CO_2/the CO_2 % vs. time for the sublittoral lab test of 2nd year (1st series)

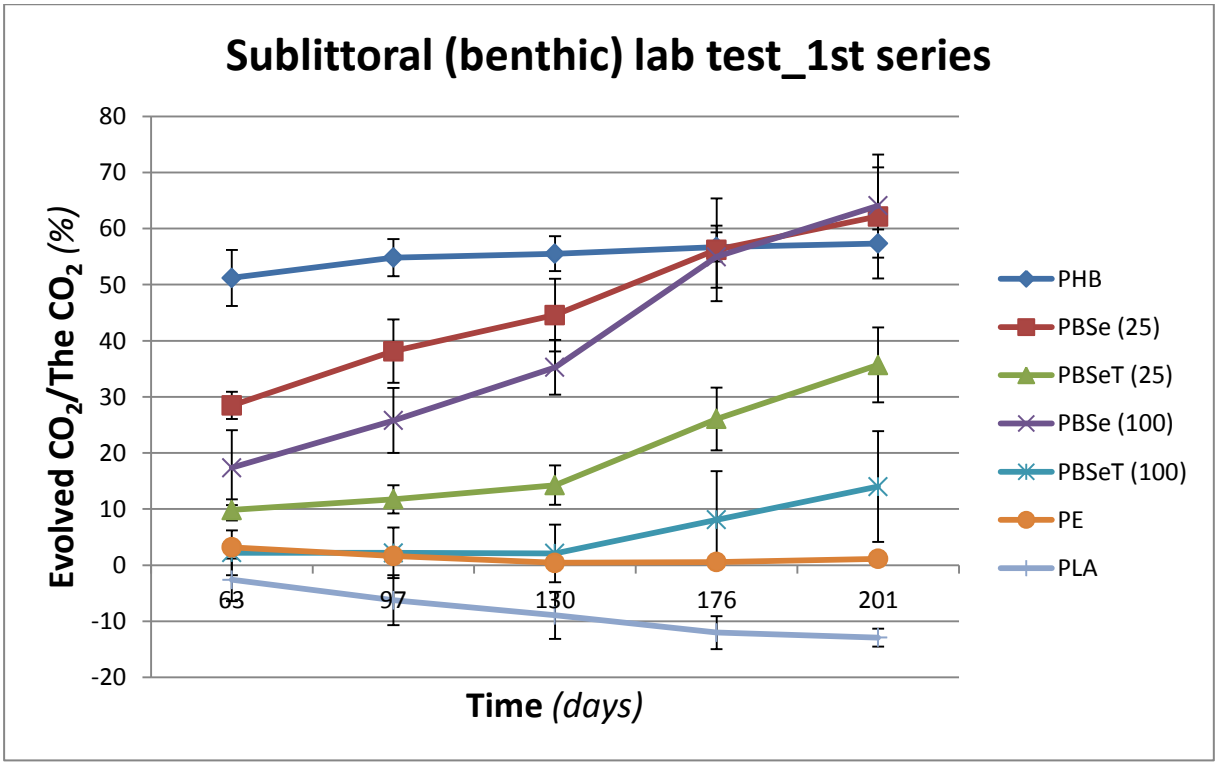


Figure 163. Average evolved over theoretical CO_2vs. time for the sublittoral lab test of 2nd year (1st series)

After 201 days of monitoring the evolved over theoretical CO_2 % for the sublittoral environment the results are summarized in Table 67.

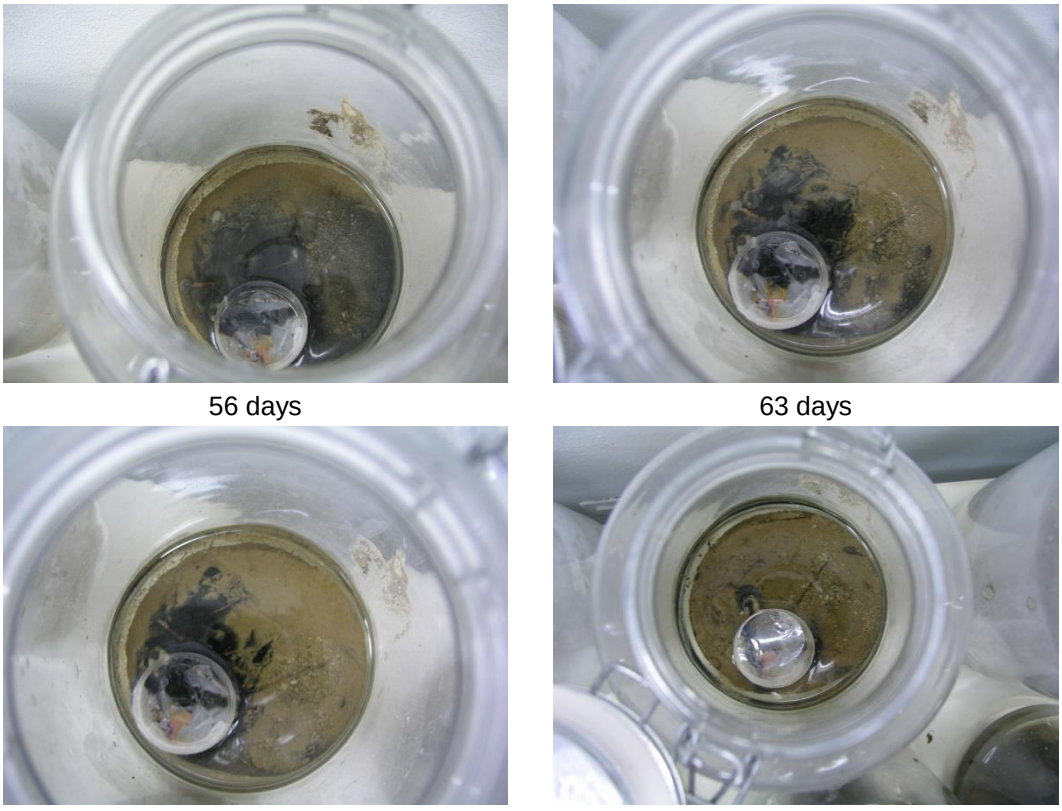
Table 67. Evolved over theoretical CO_2 % (average values and std dev) for the sublittoral environment after 201 days

	PHB	PBSe (25)	PBSeT (25)	PBSe (100)	PBSeT (100)	LDPE	PLA
Average	57.3±2.5	62.1±11.1	35.7±6.7	64.1±6.8	14.0±9.9	1.1±0.0	-13.0±1.6

As it can be concluded from the results in the first 55 days of monitoring, the production of evolved over theoretical CO_2 of PHB samples progressed fast. After this period and until the day 201 the evolved over theoretical CO_2 of PHB reached a plateau. In contrast, the PBSe samples, both the ones of 25 μm thickness and those of 100 μm thickness, present a smaller but constant evolved over theoretical CO_2 production. The PBSeT samples exhibit a behaviour similar to PBSe, but the corresponding incubation period appears longer. The LDPE shows a zero evolved over theoretical CO_2 production and the PLA samples negative values of evolved /theoretical CO_2. The behaviour of PHB samples can be explained in terms of the anaerobic activity which appears during the first stage period of fast biodegradation activity for this material, associated with consumption of the available oxygen in combination with the fact that the diffusion of oxygen to the seawater and to the sediment is limited. This leads to the formation of a black area (layer) on the sediment which appears approximately 10 days after the beginning of the experiment and which is continuously expanding and is accompanied with a high rate of evolved over theoretical CO_2 production. After this initial period, the evolved over theoretical CO_2 production remains almost constant, the plateau phase is reached and the black area is decreasing. Similar phenomena appear with PBSe and PBSeT samples at a later stage, when evolved over theoretical CO_2 production increase. An effort was made to monitor this phenomenon by photographing the reactors in different times. The results are presented in Figure 164 .

43 days

50 days

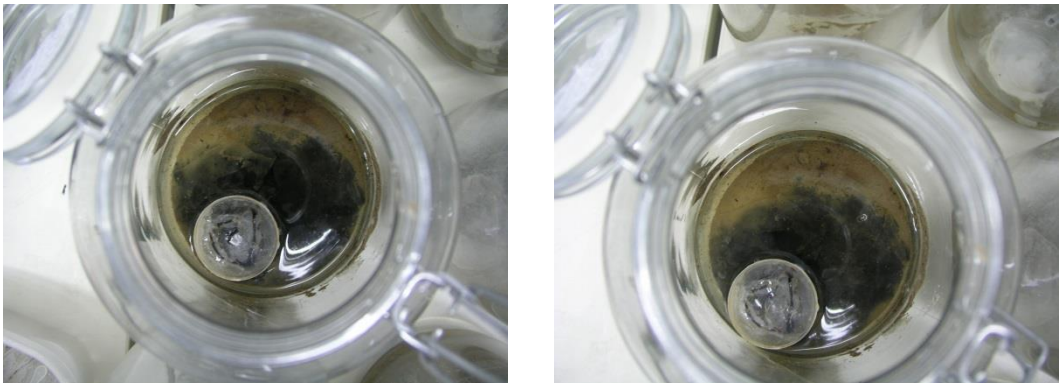


56 days 63 days

74 days 147 days

Figure 164. PHB 1 in different times of monitoring

The black region is formed firstly in the reactors with the PHB samples where it grows more rapidly than in the reactors with the PBSe samples and thereafter it disappears earlier in the PHB reactors. The comparison of evolved over theoretical CO_2 values for the PHB 1 and PBSe 1 samples is presented in Table 68.



56 days 63 days

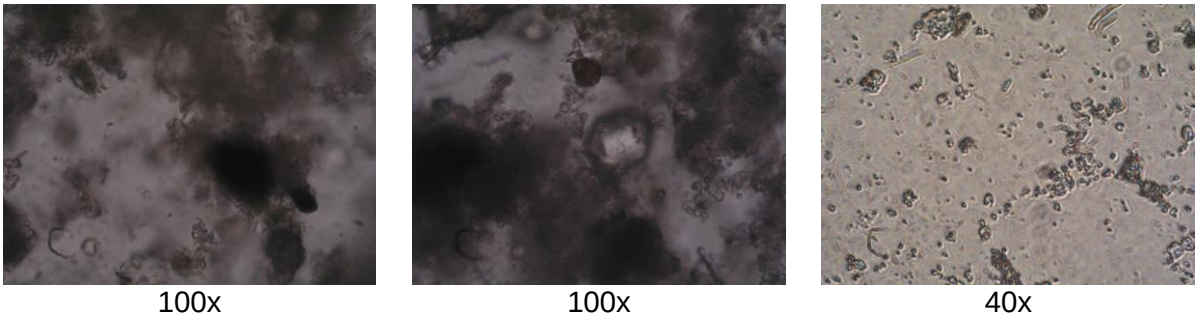
74 days 147 days

Figure 165. PBSe 1 in different times of monitoring

Table 68. Evolved over theoretical CO_2 % values for PHB 1 and PBSe 1 in different times

Days	% evolved over theoretical CO_2 PHB 1	PBSe 1
56	44.9	28.4
63	48.7	30.4
74	52.5	33.5
147	58.1	49.4
201	59.8	63.9

The observations of the black regions were also performed by means of optical microscopy and are presented in Figure 166. It appears that the black formation is made of aggregation of microorganisms.



100x 100x 40x

Figure 166. Photos from microscope of PBSe 1, 56 days of biodegradation a) 1st region, b) 2nd region and c) successive dilutions of black area in water

2nd series of benthic lab tests

The second series of experiments for the sublittoral environment were performed by using the same amount of organic carbon for each material (300 mg). In this test only PHB and LDPE samples were considered. That means approximately 600-640 mg sample for the PHB materials (47.82% organic carbon content) and approximately 350-360 mg sample for

the LDPE materials (organic carbon content 85.03%). The exact weights and corresponding organic carbon of materials are presented in Table 69. The experiment included 2 blanks, 3 PHB and 2 LDPE replicates. From the PHB replicates the PHB No. 3 was perforated. The LDPE No. 1 replicate was washed with ethanol before used. The same fertilizer was used as in the 1st series. The weight of the additional fertilizer used was calculated based on a ratio of N/C equal to 1/10 with respect to the organic carbon contained in the sample. The appropriate amount of fertilizer was dissolved in natural seawater and added to each reactor. The difference from the 1st series of sublittoral experiment was that the volume of reactors was 2L in the 2nd series. As mentioned above, 170 g of sediment were added to each reactor and the volume of natural seawater plus the natural seawater with the fertilizer added in each reactor was approximately 400 ml. The CO_2 production determined by titration was monitored for a period of 93 days. The results are presented in Figure 167Figure 161. The parameters of this experiment are presented in Table 70.

Table 69. Weights and corresponding organic carbon of materials used for 2nd series of the sublittoral test of 2nd year

Thickness (μm)	Material	Weight of sample (mg)	Organic carbon in sample (mg)
80	PHB 1	636.0	304
	PHB 2	605.6	290
	PHB 3	636.3	304
27	PE 1	351.5	299
	PE 2	365.2	310

Table 70. Parameters of the sublittoral lab test of 2nd year (2nd series)

Sublittoral test Parameters	
Reactor volume (L)	2.0
Type	Static (closed vessel)
Temperature (°C)	25-28
Sample characteristics	Piece of plastic film
Quantity of sample (mg of organic carbon)	300
Quantity of inoculum (g)	Around 170
Measurement method	CO_2 titration
Chemical reagent	KOH 0.5 N and 0.2N and HCl 0.25M
Seawater (ml)	Natural (around 400)

Nutrients: N (g)/sample TOC (g)	0.1/1.0

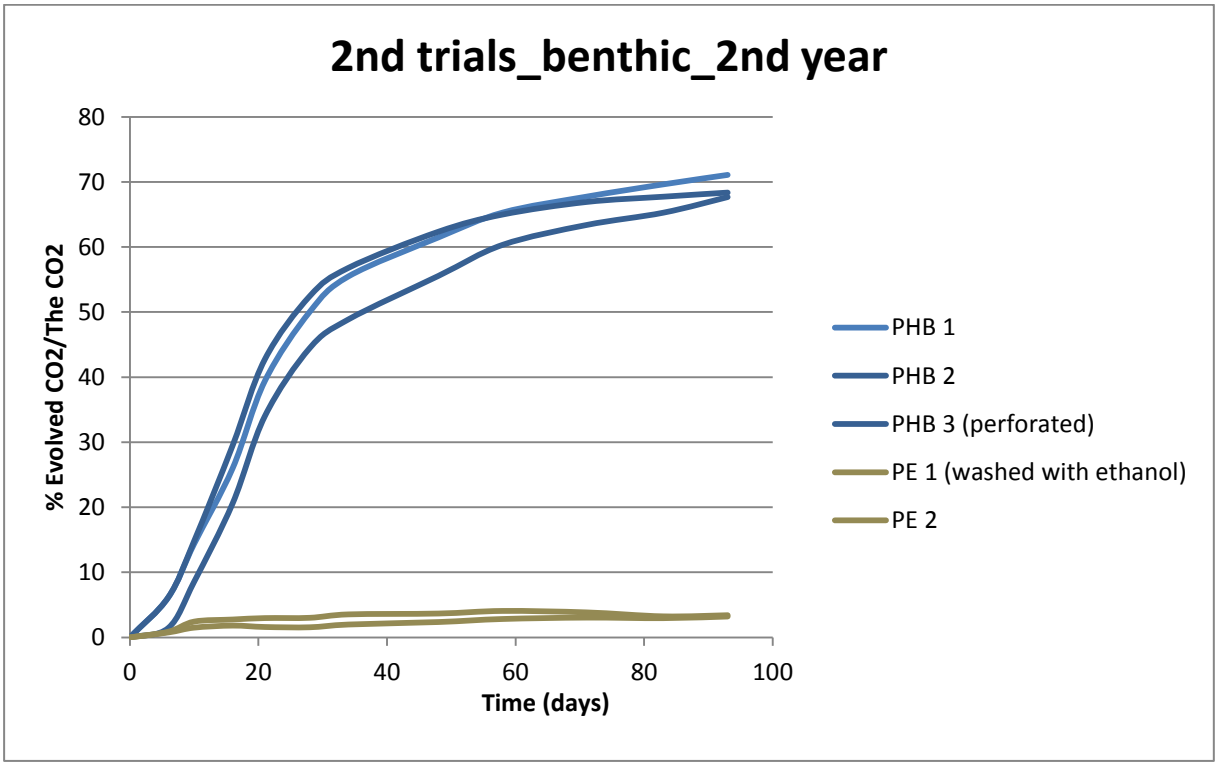


Figure 167. Evolved over theoretical CO2 % vs. time for the sublittoral lab test of 2nd year (2nd series)

Physical observations of the progress of the experiment were made by photographing the reactors in different times. Similarly to the 1st series of sublittoral experiment, the formation of black areas on the sediment was observed. The formation started on the 10th day of the experiment with a small delay for the perforated PHB and approximately on the 60th day of monitoring the evolved over theoretical CO_2 production the black areas disappeared on both PHB No.1 and No. 2 replicates and later on for the PHB No. 3 replicate (perforated material). The progress of the experiment is presented in Figure 168. After 93 days the results of values of evolved over theoretical CO_2 % are presented in Table 71.

Table 71. % evolved/theoretical CO_2 for the sublittoral environment (2nd series) after 93 days

PHB 1	PHB 2	PHB 3	PHB (average)	PE 1	PE 2	PE (average)
71.1	68.4	67.7	69.1±1.8	3.4	3.2	3.3±0.1

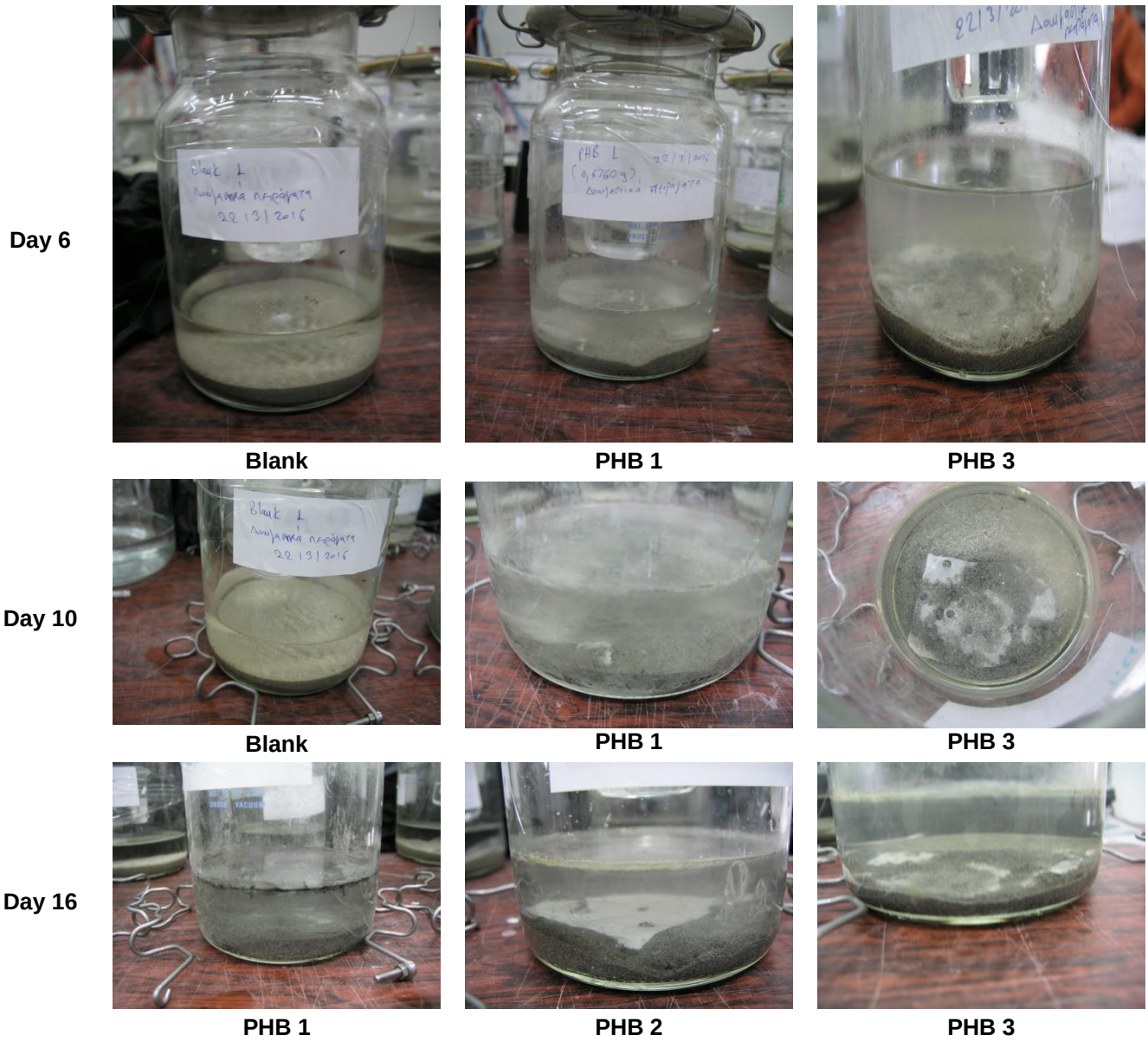


Figure 168. Images of the reactors of 2nd series of sublittoral experiment of 2nd year (a) 6th day, (b) 10th day and (c) 16th day

Analytical characterization of the black layer: Microbial evaluation of the black layer

The microorganisms present in the black layer were first investigated by a microbiological analytical test. From the result of the test the microorganisms were characterized as anaerobic due to the fact that they precipitated in the bottom of a tube containing broth where there is lack of oxygen while on the surface of the tube gas was evolved indicating the anaerobic activity of the microorganisms. The phenomenon is shown in Figure 169.

Figure 169. Microbiological evaluation of the black layer

Optical microscopy of the black layer

Sample was taken as it is shown in Figure 170 and it was observed by optical microscopy. The black area appears as a black membrane formation. When exposed to the air and dried, it becomes a mass of sand while the black colour disappears.

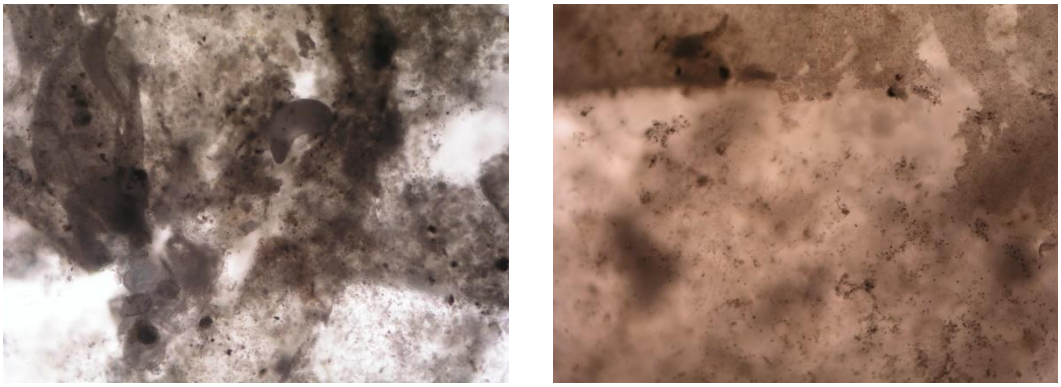


Figure 170. Black layer from the reactors and optical microscopy *Characterization of black layer by FTIR ATR (day 14)*

A sample of the black layer was taken from the reactors and was placed on paper. Consequently the black layer was characterized by means of FTIR ATR spectroscopy and the spectrum obtained was compared to the spectrum of net paper. As it is shown in Figure 171, peaks are observed in the region of 1640 cm $^{-1}$ and 1740 cm^{-1} which are absent in the spectrum of net paper. These peaks are possibly associated with biological effects and may correspond to amide groups. These peaks do not originate from the fertilizer used which includes nutrients in nitric form. The spectrum of the fertilizer diluted in seawater and placed on cellulose paper was obtained by means of FTIR ATR spectroscopy. This spectrum is presented in Figure 172 confirming that the peaks at 1640 cm $^{-1}$ and 1740 cm^{-1} are absent. The broad peak at 3300 cm^{-1} represents OH^- groups most probably produced by hydrolysis. This peak is observed for the sample of cellulose paper as well but its intensity is significantly increased for the black layer sample.

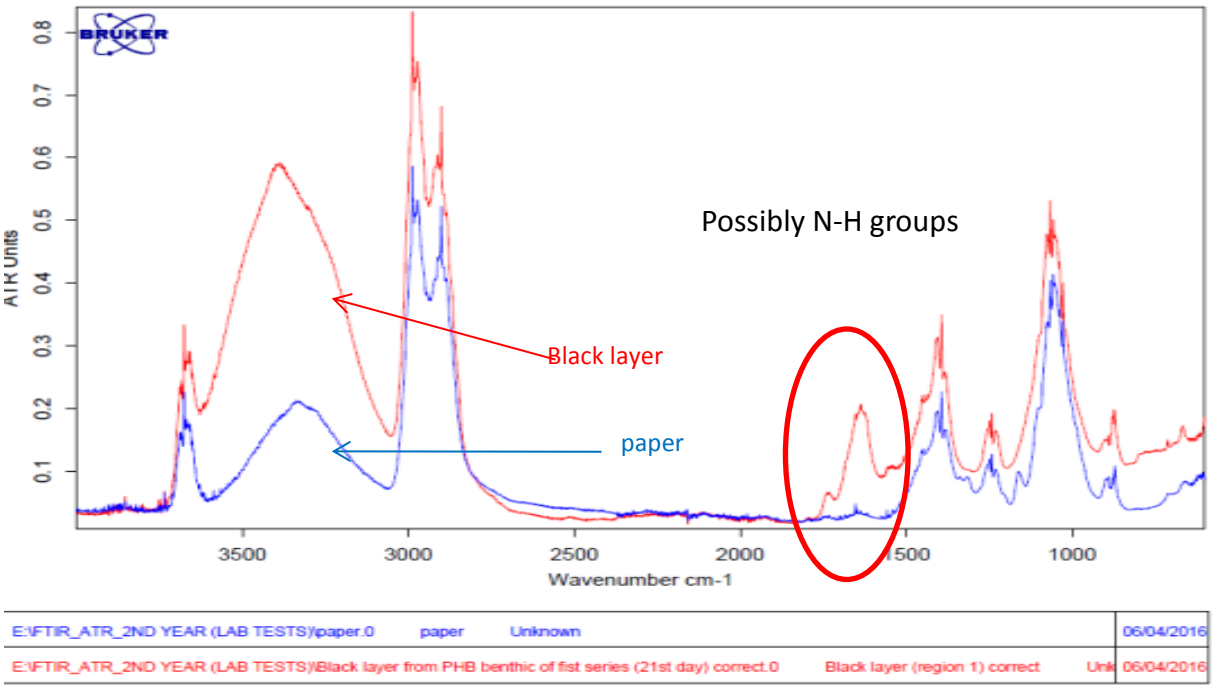


Figure 171. FTIR ATR of the black layer compared with FTIR ATR spectrum of paper

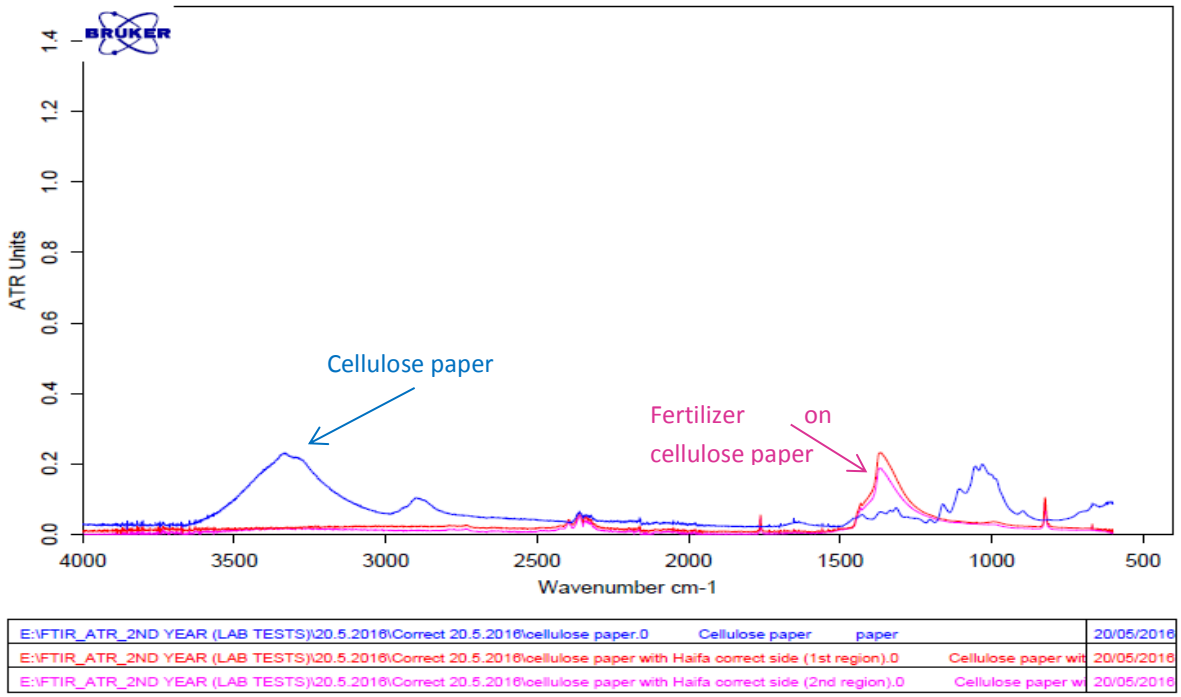


Figure 172. FTIR ATR of the fertilizer on cellulose paper compared with FTIR ATR spectrum of net cellulose paper

3rd series of benthic lab tests (system with agitation)

A 3rd series of experiments for the sublittoral test was performed aiming to overcome the problem of the black areas formation and the phenomenon anoxic conditions leading to anaerobic phenomena. For this reason agitation was added to the reactors. The experimental set up is presented in Figure 173 and the parameters of the test are presented in Table 72. The parameters of this test are in general similar to the other sublittoral tests except the addition of agitation to the system. Two replicates of PHB samples were used and one blank. The exact weights of the samples and the corresponding organic carbon content are presented in Table 73. The biodegradation was monitored for a period of 44 days and the results are presented in Table 74 and in Figure 174.

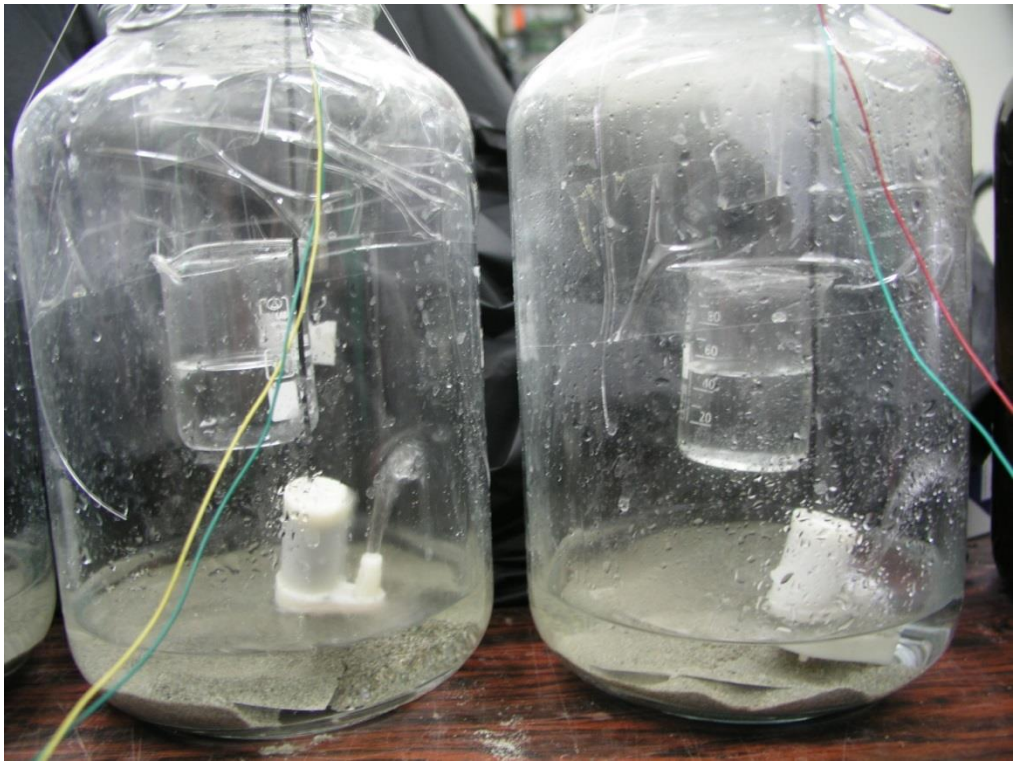


Figure 173. Experimental set up of the 3rd series of sublittoral test

Table 72. Parameters of the sublittoral lab test of 2nd year (3rd series)

Parameters	
Reactor volume (L)	4.0
Type	With agitation (closed vessel)
Temperature (°C)	25-28
Sample characteristics	Piece of plastic film
Quantity of sample (mg)	300
Quantity of inoculum (g)	Around 170
Measurement method	CO_2 titration
Chemical reagent	KOH 0.5 N and 0.2N and HCl 0.25M
Seawater (ml)	Natural (around 400)
Nutrients: N (g)/sample TOC (g)	0.1/1.0

Table 73. Weights of samples and corresponding organic carbon for the 3rd series of sublittoral test

Material	Weight of sample (mg)	Organic carbon in sample (mg)	Organic carbon in sample (mg)
PHB 1	628.7	300.6	300.6
PHB 2	629.1	300.8	300.8

Table 74. Analytical results of the 3rd series of sublittoral test (% evolved over theoretical CO_2 vs time)

Time Days	% Evolved over theoretical CO_2 (CO_2 evolved/CO_2 theo) PHB 1	PHB 2
0	0.0	0.0
8	14.3	12.0
14	28.2	22.5
17	33.1	26.9
22	40.1	36.6
30	52.3	50.7
44	60.0	65.6

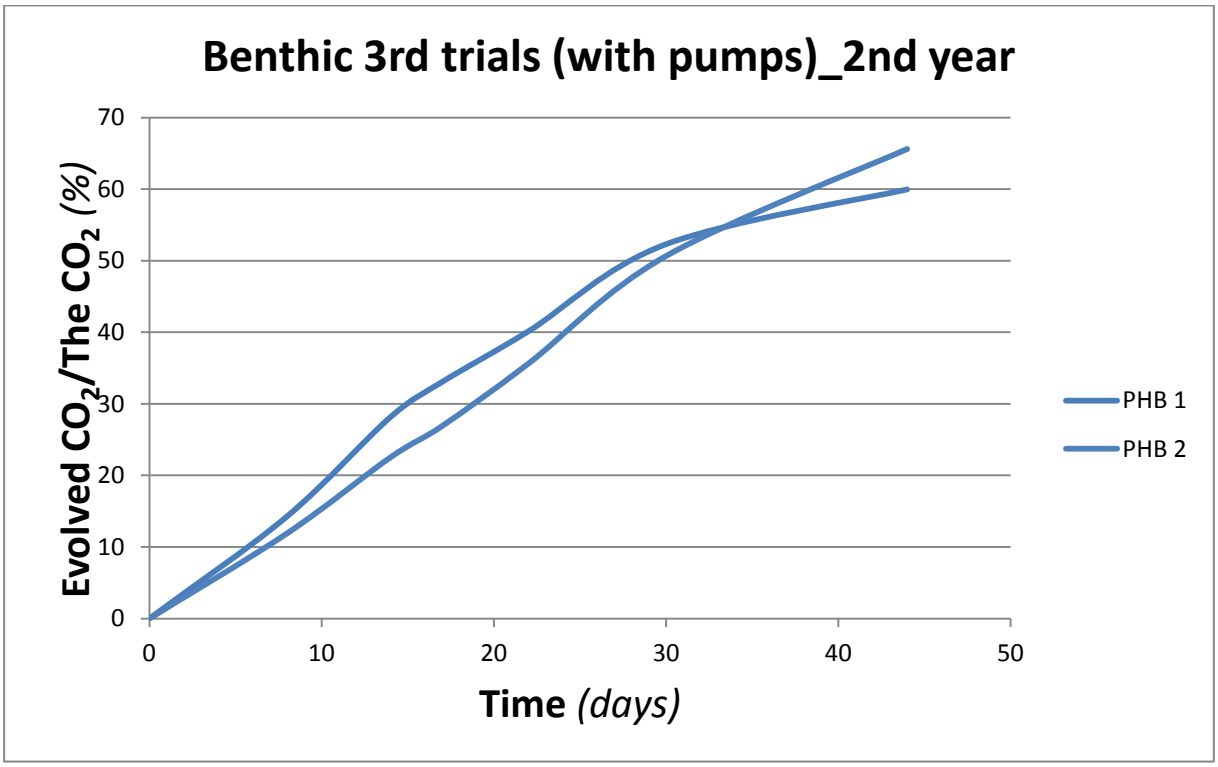


Figure 174. Evolved over theoretical CO_2 %vs. time for the 3rd series of sublittoral test

Table 75. Comparison of evolved CO_2 over theoretical CO_2 between 1st and 3rd series of sublittoral tests (static system compared to system with agitation)

1st series (static system)	PHB 1	PHB 2	PHB 3	Average	sdev
42 days	39.4	51.4	41.7	44.2	6.4
3rd series (system with agitation)	**PHB 1**	**PHB 2**		**Average**	**sdev**
44 days	60.0	65.6		62.8	4.0

Comparing the results between the static system without agitation (1st series) and the system with agitation (3rd series) the evolved over theoretical CO_2 production after 42 days for the first system is 44.2% ±6.4% while for the latter is 62.8% ±4.0%. These results are

presented in Table 75. These preliminary results suggest that the agitation in the system is able to enhance the aerobic biodegradation rate significantly, avoiding the development of anoxic conditions during the high biodegradation rate period. This is probably due to the fact that the system is aerated in a satisfactory degree and the diffusion of oxygen to the seawater and to the sediment is enhanced. Even with the aeration measures applied here the PHB had not reached a plateau phase when the tests were stopped. A negative control was not included in this test round. Therefore, more research will be necessary to clarify this aspect.

6.5.3 Biodegradation in Pelagic zone

Experimental procedure

During the first year's test, the average evolved over theoretical CO_2 % of the tested materials in the pelagic environment did not exceed 80%. A possible reason for this behaviour is the limited headspace of the bioreactors as it was discussed in section Pelagic test – closed vessel system (for the 1st year lab test results). For this reason, the testing method was modified during the second year. The new experimental procedure was as follows: Glass flasks of 4L volume were used for this experiment. The reactors were filled with 100 ml seawater and the materials corresponding to 150 mg organic carbon were placed in the reactors. In the blank reactors no material was introduced. The examined system constituted 2 blanks, 2 PHB, 2 PBSe (25μm) and 2 PBSeT (25μm). Nutrients were added to the reactors by adding "Haifa" fertilizer so to correspond to a ratio of N/C: 1/10 and F2 fertilizer so to correspond to the same ratio of N/C (1/10). The two types of fertilizer were added the one in the one of the two replicates and the other to the second. This means that 1 blank, 1 PHB, 1 PBSe and 1 PBSeT of the replicates contained Haifa fertilizer and 1 blank, 1 PHB, 1 PBSe and 1 PBSeT of the replicates contained F2 fertilizer. Stirring bars were added to the reactors which were placed on magnetic stirrers. Trapping solutions for the CO_2 produced were KOH solutions and the traps were placed inside the reactors above the seawater. The method used was based on the CO_2 determination by titration with HCl 0.25N. The experimental set up of the test is presented in Figure 175 and the parameters of the experimental set up are summarized in Table 76. The exact weights and the corresponding organic carbon of the materials used in the pelagic lab tests of 2nd year are presented in Table 77.

Figure 175. Experimental set up for the pelagic lab test of 2nd year

Table 76. Parameters of pelagic lab test of 2nd year

Pelagic test Parameters	
Reactor volume (L)	4.0
Type	Static with stirring
Temperature (°C)	25-28
Sample characteristics	Plastic specimen
Quantity of sample (mg)	200- 300 so to correspond to 150 mg organic carbon
Quantity of inoculum (ml) (natural seawater)	100
Measurement method	CO_2 titration
Chemical reagent	KOH 0.2, 0.5 and 1N and HCl 0.25 N
Nutrients: N (g)/sample TOC (g)	Haifa fertilizer 1/10 (N/C), F2 fertilizer 1/10 (N/C)

Table 77. Weights and corresponding organic carbon used for the pelagic lab test of 2nd year

Thickness (μm)	Material	Weight of sample (mg)	Organic carbon in sample (mg)
80	PHB 1	310.7	148.6
80	PHB 2	309.5	148.0
25	PBSe 1	235.6	153.7
25	PBSe 2	229.3	149.6
25	PBSeT 1	233.0	152.0
25	PBSeT 2	233.0	152.0
30	LDPE	177.0	150.5

The evolved over theoretical CO_2 %was monitored for a period of 113 days. The analytical results are presented in Figure 176.

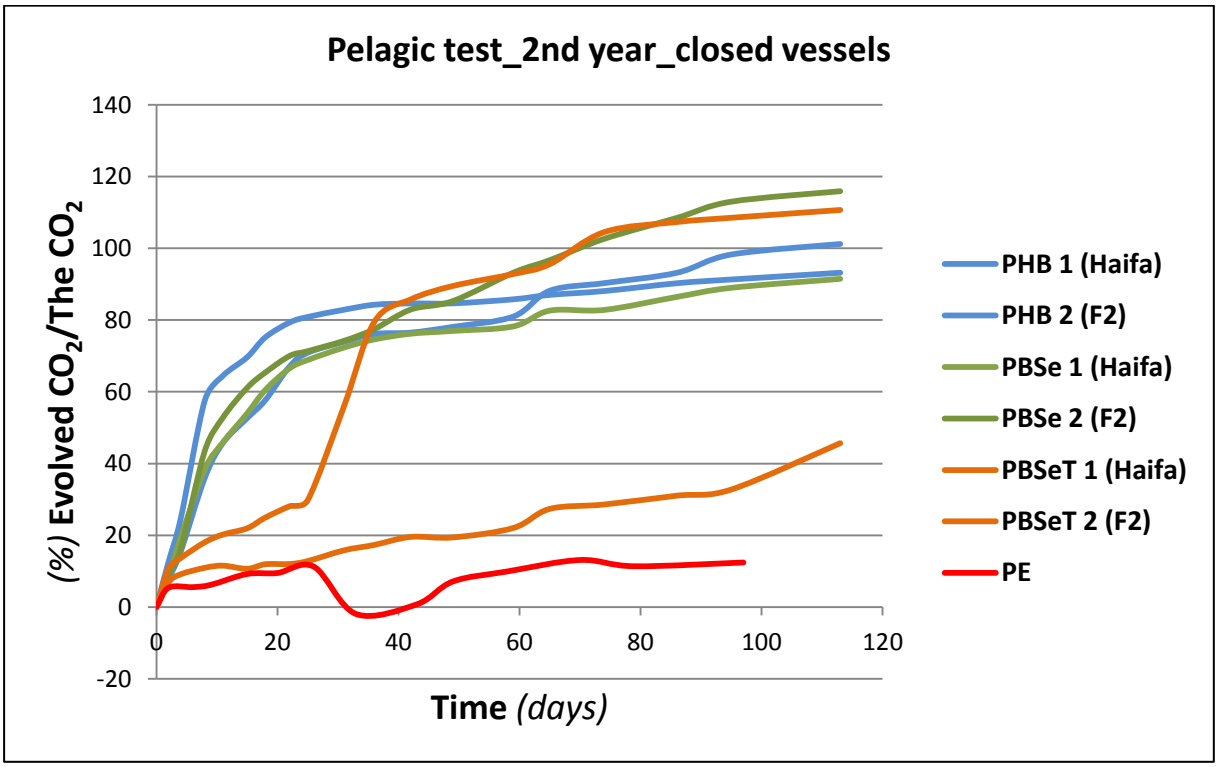


Figure 176. Evolved CO_2/The CO_2 (%) vs. time for the pelagic lab test of 2nd year

As it can be seen from the results in Figure 176, after the 25th day, the evolved over theoretical CO_2 % of PBSeT 2 (with F2 fertilizer) abruptly increased significantly and the material almost disappeared. The difference between the two vessels with the same material but different fertilizer is shown in Figure 177. After 74 days the evolved CO_2 from PBSe 2 (with F2 fertilizer) and from PBSeT (with F2 fertilizer) exceeded 100%. After 113 days the

evolved CO_2 by PBSe 2 (with F2 fertilizer) exceeds 100% in a percentage 10 % and the evolved CO_2 by PBSeT 2 (with F2 fertilizer) exceeds 100% in almost the same percentage. At the same time the evolved CO_2 by LDPE seems to have an error value of approximately 10%. These errors are analogous, therefore, the method needs to be optimized in order to avoid such deviations from the theoretical values.

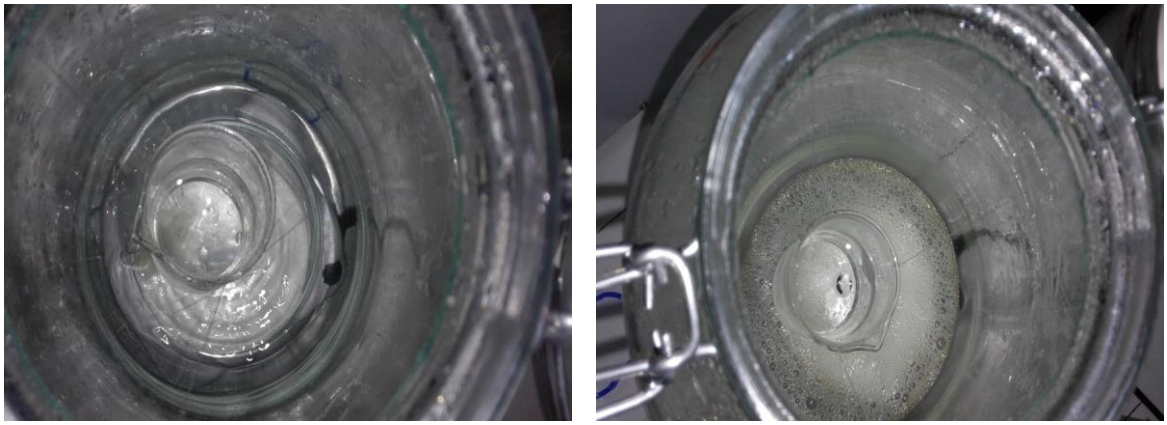


Figure 177. (a) PBSeT (with Haifa fertilizer), (b) PBSeT (with F2 fertilizer) (32nd day of experiment)

Figure 177 shows that in the vessel which contained the PBSeT No. 2 (with the F2 fertilizer), the material has disintegrated and foam appeared in the vessel. From this reactor, water samples were taken and studied by optical microscopy. The morphology of some regions of the sample is presented in Figure 178 showing the growth of clusters of micro-organisms.

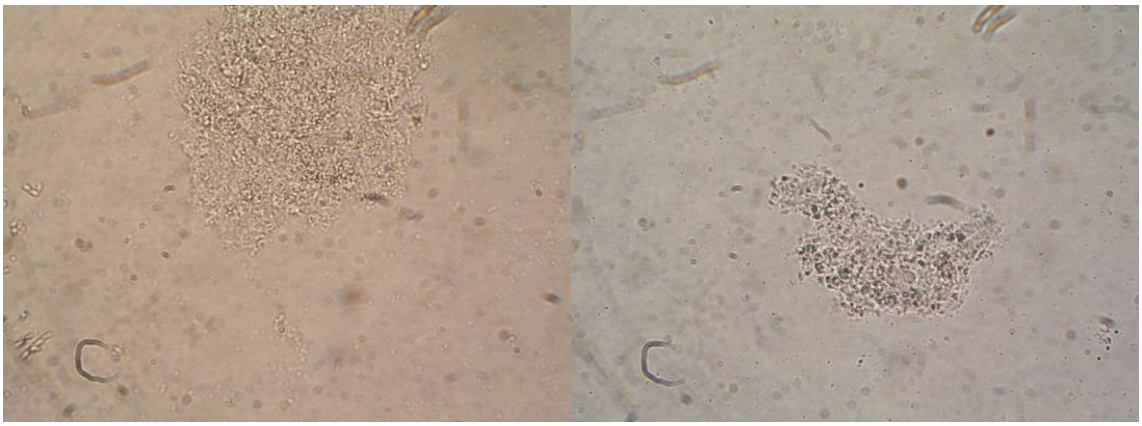


Figure 178. Sample from the vessel with the PBSeT 2 (with F2 fertilizer) after 32 days of experiment (a) 40x (b) 40x

7 General discussion

7.1 Introduction

The main objective of this task in work package 5 is the development of marine biodegradation testing methods for bio-based materials. For this reason, three different laboratory testing methods have been evaluated, each focusing on a different set on environmental conditions that may occur in the marine environment, representing the benthic, eulittoral and pelagic zones. Five different laboratories performed these tests following a common protocol. The same protocol was followed using two different inoculums (i.e. sampled from two different Mediterranean locations). The tests were evaluated using four different plastic materials, including a material that is known to be persistent (not biodegradable) in the environment (the negative control, LDPE), a material that is known to be readily biodegradable in many environments (the positive control, PHB), and two custom made materials that were expected to have an intermediate biodegradation rate (the aliphatic polyester PBSe and the aliphatic-aromatic copolyester PBSeT). Finally, the tests were repeated in two consecutive years, including some modifications based on the findings in the first series of tests.

This general discussion is divided in three sub-sections, each covering the evaluation of one test method, i.e. representing the environmental conditions ofthe benthic, the eulittoral and the pelagic zones, respectively. For the different tests the “ultimate” biodegradation was calculated and reported followed by the respective standard deviation. In each sub-section the discussion of the remarkable results concerning the first year are addressed as the suggestions and improvements coming from the plenary meetings. In the same way the second year results are explained and compared with the effect of the modifications made to the first year test method. A general conclusion with regard to the test protocol (level of maturity, suitability, and suggestions for further modification) are presented.

7.2 Test method: Benthic environment – biodegradation at the sediment-seawater interface

7.2.1 Year 1 results

In Table 78 a summary of the biodegradation results in the Benthic environment (both inocula: Italian and Greek) is reported. The test materials appear to biodegrade in the benthic environment (where PHB is degraded more efficiently than PBSe and PBSeT) and all laboratories involved were able to measure their biodegradation.

Table 78. General summary of Benthic environment results year 1

Year 1 Benthic (seawater/sediment interface Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	181	274		266	
PHB	249.4 (±16.8)	104.5 (±22.9)	-	58.9 (±31.4)	-
PBSe	181.2 (±46.9)	84.92 (±35.4)	-	94.42(±12.0)	-
PBSeT	185.6 (±26.3)	59.21 (±27)	-	53.6 (±24.7)	-
LDPE	98.2 (±15.3)	-	-	15.2 (±12.1)	-
Year 1 Benthic (seawater/sediment interface Elba) **Biodegradation %**					
Test duration (days)	182		331	259	280
PHB	234.4 (±20.7)	-	82.4 (±6.9)	109.2 (±20)	87.6 (±0.4)
PBSe	117.4 (±20.5)	-	76.1 (±2.3)	74.08 (±5.5)	89 (±20.9)
PBSeT	116.9 (±12.2)	-	71.03 (±4.3)	103.5 (±23.1)	81.1 (±1.7)
LDPE	93.6 (±8)	-	-2.02 (±1.4)	4.3 (±6.3)	2.3 (±3.5)

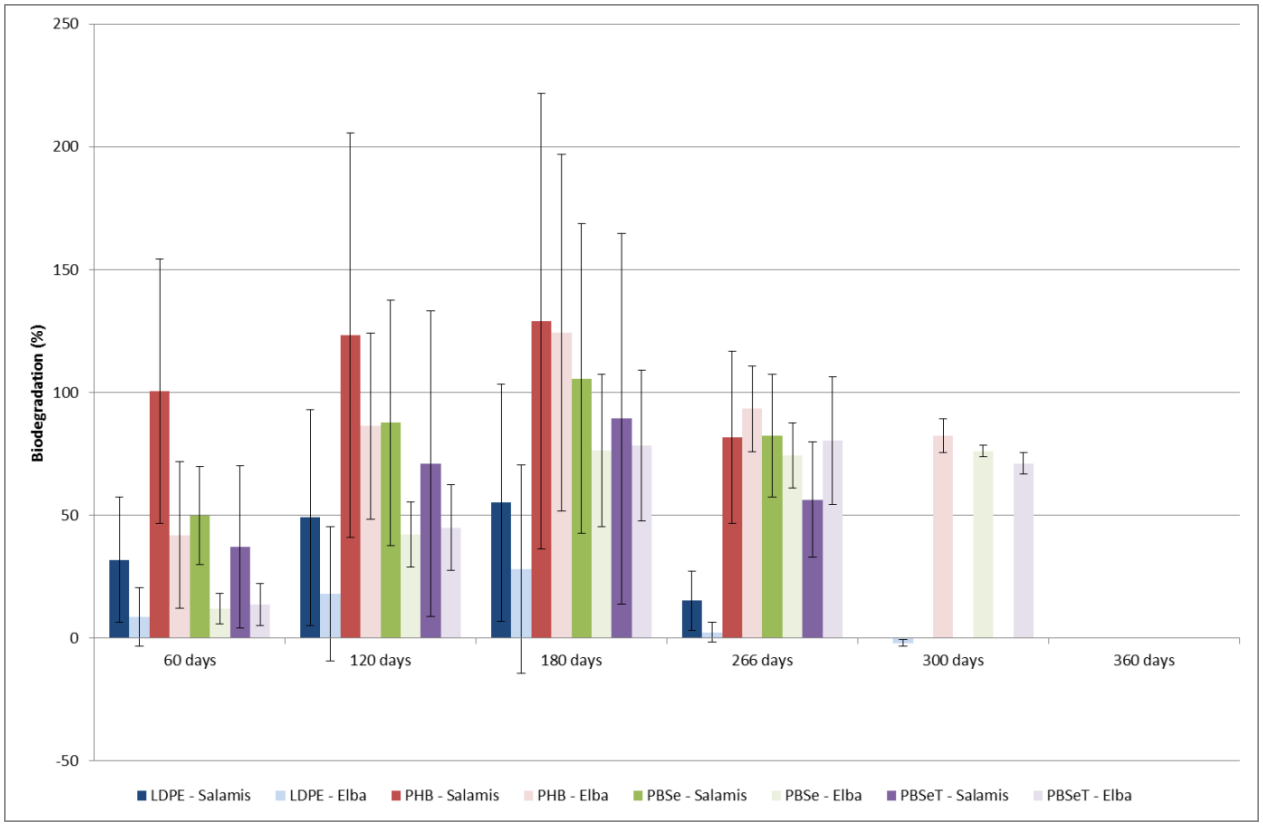


Figure 179. Results Sediment/Seawater interface test - year 1; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

The results showed that there were large differences between the actual biodegradation results obtained in the different labs (Table 79, Figure 179) although in every lab the biodegradation trend observed for the different test items was similar: i.e. PHB>PBSe and PBSeT>LDPE. Following was observed in year 1:

- Large standard deviations between triplicate measurements for some of the labs but also on an inter-laboratory level, e.g. when comparing the PHB biodegradation.
- Biodegradation, in some cases, exceeded 100% (result not reliable)
- Biodegradation of LDPE (negative control) was registered (CO_2 evolution higher than blank), while samples remained physically intact (weight balance after termination of the tests also indicated that no biodegradation occurred)
- Differences between the results obtained by the two inocula for the same test material (e.g. PBSeT)
- Differences in lag phases among triplicate bottles of the same test set. This was most obvious for PHB.
- Relative long test time (about 180 days) required for (complete) biodegradation of positive control. Longer test times imply larger standard deviations in biological tests, because typically variations in microbial composition start to develop immediately

after the start of the experiment. If a test is run for longer periods of time, these differences could become more pronounced.

These observations could be explained by:

- Difference in sediments at different location: the biodegradation relies on the presence of suitable microorganisms in the sediment. This microbial population may be different at different locations. Also the time between sampling the sediment and the actual application, the storage method of the sediment after arrival in the lab until further use, the pre-filtering or sieving of the sediment prior to use in the test and further handling may influence the microbial activity. Furthermore, dry and organic matter contents may be different between locations. This may affect the microbial composition, but also the activity, the background CO_2 production and whether the microorganism will start to biodegrade the test items (in case of surplus of other organic matter present);
- Occurrence of anaerobic zones in the sediment is evident from the “black zones” temporarily) formed in some of the reactors. This effect was observed in reactors containing PHB, which is the more readily biodegradable material, tested at high thickness (100 micron). The effect was not observed with the other materials tested in the concentration indicated in the protocol. The formation of the black zones could be related to a decrease of available oxygen and the subsequent formation of an anoxic area, leading to reduction of sulphate in the seawater and formation of sulphide salts, which is usually associated with the formation of black precipitates. When the biodegradation activity decreased the colour of the sediment became “normal” suggesting that the reduced molecules were oxidized again (no experimental data are available to support this theory) and/or that the oxygen supply was higher than the amount needed for sulphide oxidation and aerobic biodegradation;
- The relatively small amount of CO_2 produced as a result of biodegradation of the test items as compared to the endogenous CO_2 production in a blank without test items (e.g. Figure 180 for Elba sediment in year 1). This is important because the biodegradation of a test item is calculated as the difference in CO_2 production in the presence and absence of a test item;
- The long test time was probably related to the relatively low amount of test item defined in the protocol and used in its physical form (film);
- Although all labs are well equipped and experienced with the evaluation of biodegradation a certain percentage of the error will be caused by the analytical methods used;
- In some cases the laboratories adopted different set-ups to perform the test due to the equipment availability (i.e. incubating temperature, reactors volume etc.) or different systems to record the biodegradation rates (O_2 consumption instead CO_2 production).

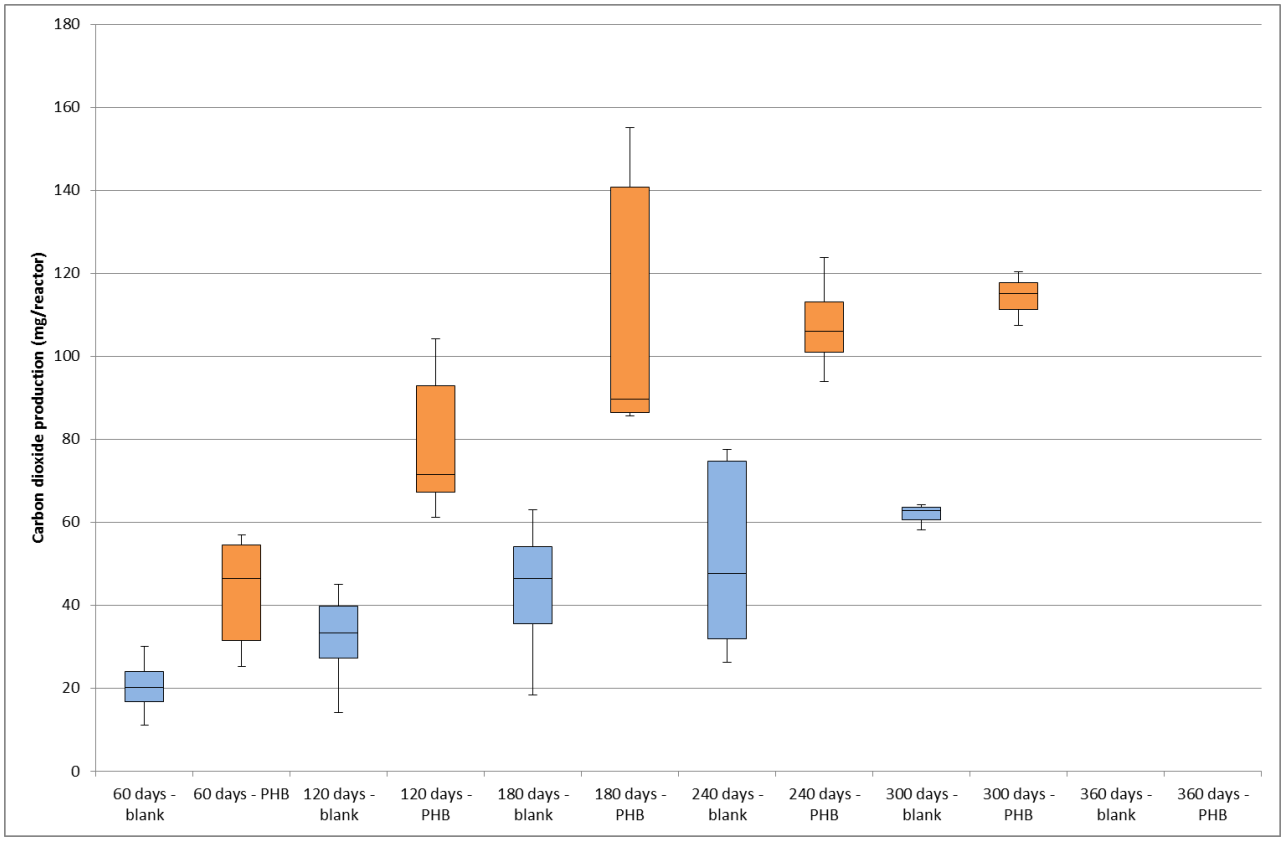


Figure 180 Results Sediment/Seawater interface test - Elba Year 1; CO_2 production in blank reactors (blue) and positive control (PHB) reactors (orange)

7.2.2 Year 2 results

For the second year testing, the method was modified to lower the background CO_2 production in the blanks. Two different pre-treatments were evaluated: (1) the sediment was mixed (50/50) with the same sediment after calcination (treatment at 550°C for 4h to remove the endogenous organic matter), and (2) the sediment was pre-incubated for 7-10 days at 20-28°C under dynamic aerobic conditions and submitted to intermitted gentle mixing to reduce the amount of biodegradable organic matter prior to use in the test.

The measures taken for the set-up of the test had a positive minor effect on the reproducibility between labs and also with respect to the standard deviation among triplicates (Figure 181 and Table 79) but were not able to solve totally the problems observed during the first year. Nevertheless, the overall biodegradation measured by all labs did not exceed 100% (which was an improvement compared to year 1) and also the CO_2 production in the reactors containing LDPE, in general, was more in line with expectations (production similar to the blank).

Besides, the results show the following:

- Data obtained incubating the reactor at 20°C (LeAF) were characterized by a lower biodegradation speed (as expected).
- Data obtained during the second year using the O_2 consumption instead the CO_2 production measurements (BASF), showed a slight decrease (about 10% for all samples) in levels of biodegradability in comparison to the results of the first year. No modification in the test method was performed.
- The possibility to increase the quantity of test items was investigated during the second year by AUA. Their approach highlighted that also without any pretreatment to the sediment but only adopting a higher test item concentration and an adequate addition of nutrients, it is possible to obtain reliable biodegradation results.
- The pre-treatment based on diluting sediment with 50% calcined sediment had the expected result, the endogenous CO_2 production was substantially lower in year 2 when compared to year 1 (see Table 80). Unfortunately, not all laboratories could adopt this strategy (only OWS and Novamont tried this pre-treatment). Data presented concerning the endogenous CO_2 reduction obtained, refer to the Elba sediment (but similar reduction was observed also with Salamis sediment, data not shown). During the first 240 days of incubation, the blank CO_2 reduction average was between 50 and 70% (Table 80). The results reported in Table 79 show an overall decrease of the biodegradation level (only few data are over 100% of biodegradation). The sediment pretreatment based on mixing with sediment after calcination resulted in a substantial reduction of the CO_2 production and in most of the cases a lower standard deviation. This fact is not directly correlated to a decrease of inoculum biodegradation activity. Only in some cases a reduced inoculum biodegradation capacity was observed (Year 2, Salamis, Novamont data Figure 95). This pre-treatment leads to a reduction of the sediment organic matter but in some cases also to an increase of the pH (9 or higher) and such a pH level could influence the microbial consortium and consequently the biodegradative capacity. This increase of pH during the calcination process is due to the transformation of carbonates in the sediment to oxides of calcium that have basic reaction (this was proven in laboratory). This fact resulted in a clear slowdown of the biodegradation curves. To overcome this problem it is suggested to dilute the sediment directly with silica sand (an example of sand usable is reported in ISO 11268-1). Also pre-treatment by dynamic incubation of the sediment under a continuous air flux and with a gentle mixing performed for 7-10 days has been tested but unfortunately this did not lead to the same reduction of volatile solids content and consequently the endogenous CO_2 production. This was confirmed in a separate experiment carried out at the laboratory of Novamont (chapter 6.1.2). Novamont performed this test in duplicate adopting in the parallel set the other pre-treatment suggested (sediment flushed and mixed for 7-10 days prior to use) in order to compare the results and to obtain valuable information concerning the best pre-treatment to include into the standard method. The test was repeated without other changes into the protocol and for both inocula. In Table 81 the results were reported. Biodegradation results are characterized by a higher standard deviation

compared to the results obtained with the calcined mix sediment. The LDPE showed the same overestimation of CO_2 as happened during year 1. These biodegradation data are related to a general sediment overproduction of CO_2 and indicated clearly that the pre-treatment adopted was not able to solve this problem.

Table 79. General summary of Benthic environment results year 2

Year 2 Benthic (seawater/sediment interface Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	227	201	233	245	
PHB	71.4 (±70.7) Replicates: 103.4% 120.4% -9.6%	57.3 (±2.5)	61.1 (±12.9)	60.0 (±39.4)	-
PBSe	21.4 (±25.2)	62.1 (±11.1)	50.2 (±1.7)	35.6 (±3.9)	-
PBSeT	7.5 (±28.1)	35.7 (±6.7)	55.1 (±5.7)	25.2 (±9.9)	-
LDPE	-9.5 (±6.2)	1.13	2.5 (±7.4)	2.6 (±3.6)	-
Year 2 Benthic (seawater/sediment interface Elba) **Biodegradation %**					
Test duration (days)	180			310	220
PHB	37.1 (±26.8)	-	-	102.4 (±9)	69.2 (±1.5)
PBSe	-25.6 (±26.5)	-	-	95.3 (±13.4)	74.3 (±2.8)
PBSeT	-46.2 (±5.9)	-	-	101.4 (±5.9)	68.7 (±1.1)
LDPE	-27.4 (±23) Replicates: -41.8% -39.5% -0.9%	-	-	2.3 (±1.8)	-1.1

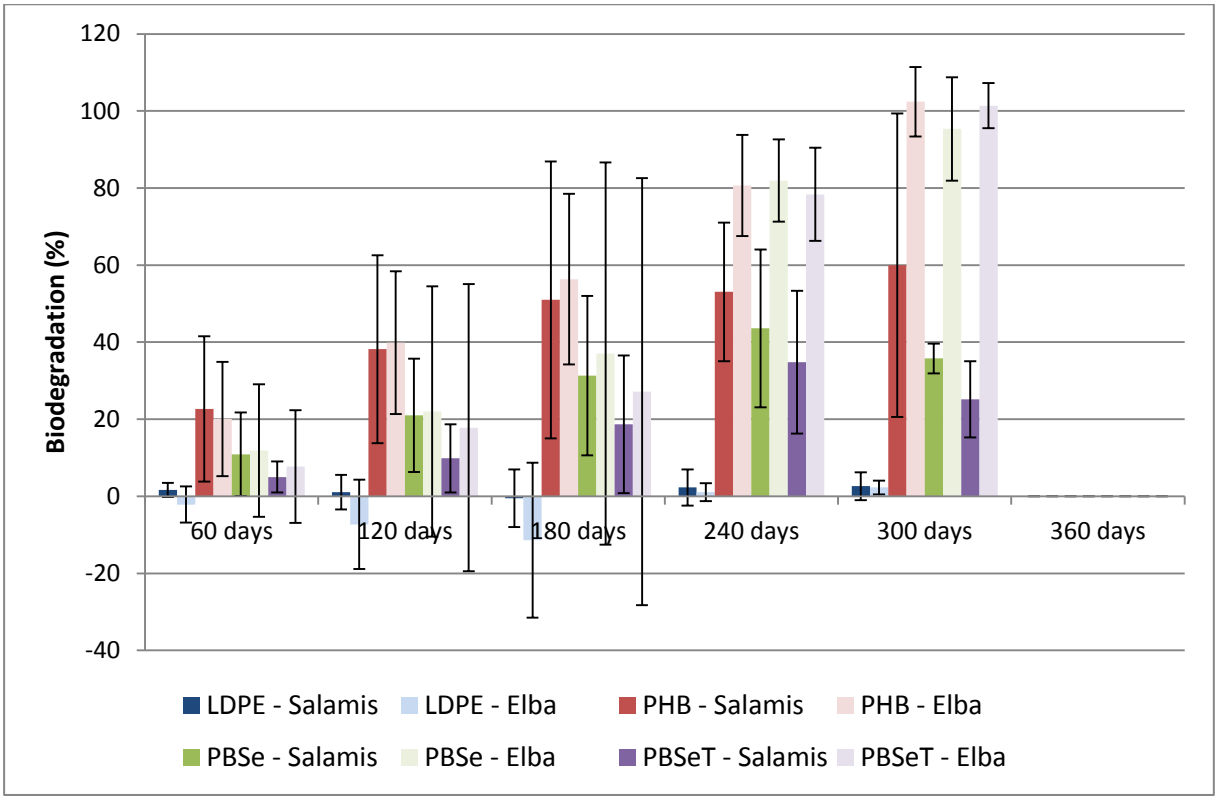


Figure 181. Results Sediment/Seawater interface test - year 2; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

Table 80. Elba: CO_2 endogenous production (based on 30 g of wet sediment)

Year 1	60 days	120 days	180 days	240 days
Novamont	21.69 ± 0.33	37.72 ± 0.99	52.33 ± 0.38	75.71 ± 1.6
OWS	29.78 ± 0.27	44.05 ± 0.90	59.81 ± 2.70	
Average	25.73	40.89	56.07	75.71
Year 2				
Novamont	7.40 (± 0.88)	10.58 (± 0.77)	15.73 (±1.79)	22 (± 1.86)
OWS	16.29 (± 1.17)	33.81 (±2.39)	48 (± 8.66)	
Average	11.85	22.20	31.87	22
CO_2 reduction (%)	53.94	45.71	43.16	70.94

Table 81. Biodegradation % obtained using sediments (both) pre-treated by air flux (year 2, Novamont results)

Test material	Biodegradation % (Salamis sediment)	Biodegradation % (Elba sediment)
	245 days	308 days
LDPE	21.19 (±10.54)	19.75 (±14.86)
PBSe	106.12 (±10.92)	93.71 (±18.26)
PBSeT	97.70 (±24.85)	100.29 (±6.51)
PHB	98.49 (±4.76)	128.65 (±30.91)

7.2.3 Conclusions

Differences are still observed in the test results obtained with the benthic environment in Year 2 although overall results in the different laboratories were more comparable in Year 2 when compared to Year 1 and the CO_2 production in the LDPE reactors was more in line with the expectations.

To improve and refine the test methods measures were discussed :

- Shorten the required test time by e.g. improving conditions in the test by mixing contents (while maintaining a sediment/seawater interface) or administering the test items in powder form or by adding nutrients
- Increase signal to noise ratio by increasing the test item concentration
- Refine the sediment pre-treatments (dilution with calcined sediment or silica sand)
- Perform the test using a sediment with a well-defined upper limit in the amount of organic matter content in order to avoid the CO_2 overproduction problem
- To adopt solutions that support the oxygen diffusion in order to avoid anaerobic situations i.e. increase the volume of the reactors, decrease the thickness of sediment layer.

These measures need to be tested to assess their effect on the outcome.

Concluding it can be stated that it is not that easy to obtain clear unequivocal results from this test method but a lot of progress was made by the large number of tests performed during these two years. Further experiments should be done following the suggestions reported in this chapter in order to obtain a fully reliable test method.

7.3 Test method: Eulittoral environment – biodegradation in wet sediment

7.3.1 Year 1 results

In Table 82 the results of Eulittoral environment are reported. In general, the results appear to be more in line with expectations and more reliable than the benthic test. Obviously, there are less variables in this test compared to the benthic test as the only biological determinant here is the sediment (as compared to sediment and seawater in the benthic test). The test materials highlighted the same biodegradative behaviour than the benthic environment: PHB showed the highest biodegradation followed by the polyesters (PBSe and PBSeT). Generally, the test method did not highlight the overproduction of CO_2 as happened, in some cases, for the benthic test. In this environment the CO_2 production in the LDPE series was very similar to the blank series (as expected) and a biodegradation higher than 100% for PHB material generally was not observed. Only in the Novamont results one replicate for each sediment has exceeded 100%.

Table 82. General summary of Eulittoral environment results year 1

Year 1 Eulittoral (wet sediment Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	425	372		368	
PHB	55.3 (±8.2)	85.0 (±3)	-	86.5 (±37.4)	-
PBSe	18.7 (±10.4)	75.3 (±5.9)	-	60.9(±1.7)	-
PBSeT	11.9 (±7.8)	49.8 (±8.4)	-	55.2 (±2.2)	-
LDPE	5.7 (±4.9)	-	-	11.8	-
Year 1 Eulittoral (wet sediment Elba) **Biodegradation %**					
Test duration (days)	425		331	329	220
PHB	95.8 (±4.1)	-	66.8 (±13.5)	92.4 (±27.7)	52.3 (±18.1)
PBSe	23.4 (±3.3)	-	34.6 (±7.8)	41.3 (±14.7)	27.4 (±26.3) Replicates: 49.7% 34.2% -1.6%
PBSeT	23.3 (±6.7)	-	33.1 (±1)	62.6 (±2.5)	26.1 (±19)
LDPE	3.6 (±4.1)	-	-1.0 (±1.6)	0.0 (±0.5)	0.9 (±3.4)

In Figure 182 the biodegradation trends obtained using both inocula are reported. The graph highlights as a regular increasing trend of biodegradation was obtained and that there was a general variability between the results obtained by the different partners. This aspect should be improved by modification of the test method. It is remarkable that the duration of

the test is very high while complete biodegradation has not been achieved at the end of 360 days of duration.

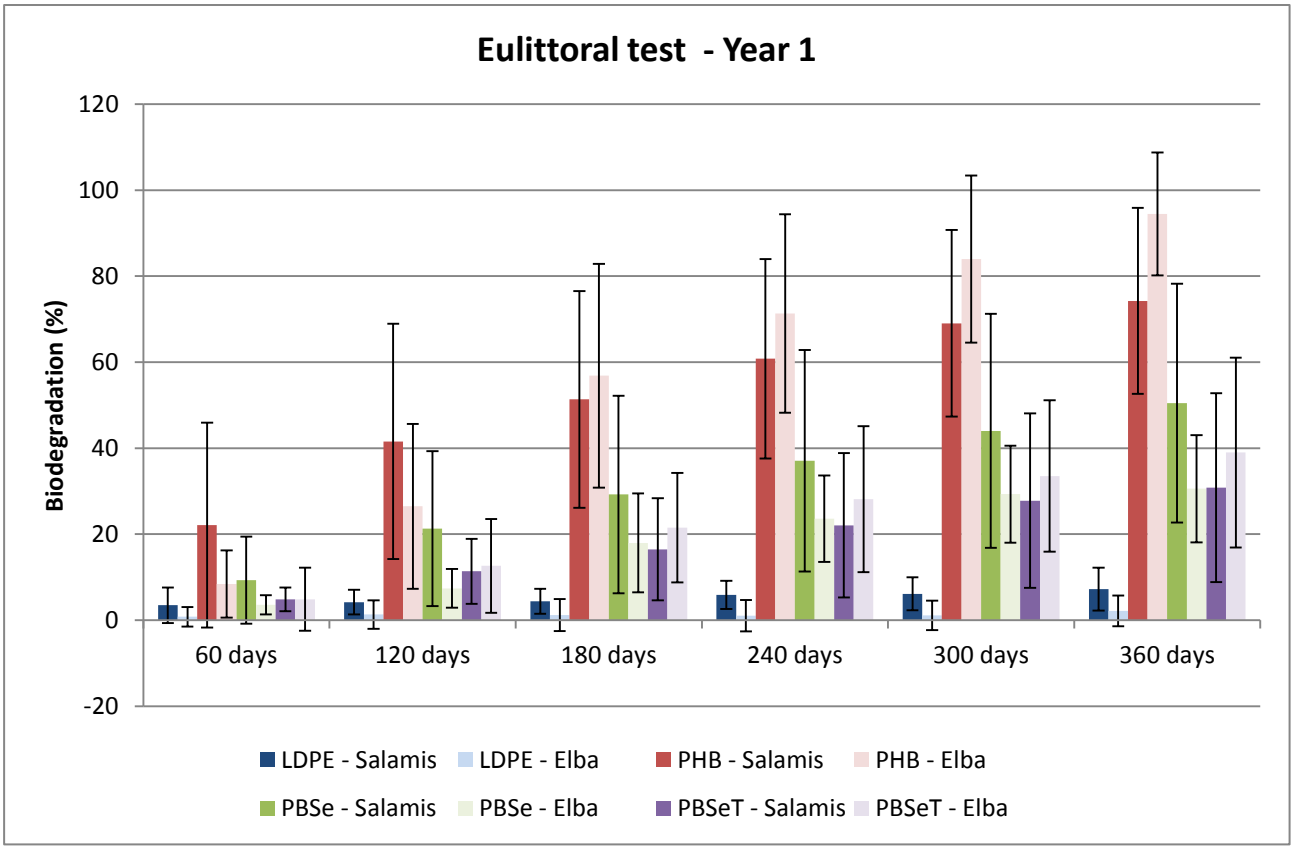


Figure 182. Results eulittoral (wet sediment) test - year 1; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

The above results permitted to do following observation regarding the test method applied:

- No clear differences between the two inocula
- Differences in lag phases among triplicate bottles of the same test set.
- Very long test time required for (complete) biodegradation. Longer test times imply larger standard deviations in biological tests, because typically variations in microbial composition start to develop.
- Possible deficit of nutrients

These observations could be explained by:

- The relatively small amount of CO_2 produced as a result of biodegradation of the test items, compared to the endogenous CO_2 production in a blank without test items (e.g. Figure 183 for Elba sediment in year 1);
- The long test time was probably related to the relatively low amount of test item used in the protocol and its physical form (film)

- Although all labs are well equipped and experienced with the evaluation of biodegradation, some of them for the first time used this methodology. Furthermore a certain percentage of the error will be caused by the analytical methods used

Data concerning the CO_2 production (Elba sediment) were reported graphically in Figure 183. The graph highlighted that there is a first overlapping of the endogenous CO_2 with the PHB test item (day 60). When the biodegradation of PHB started, the CO_2 production was higher to the endogenous ones and this separation remained stable for all the test duration.

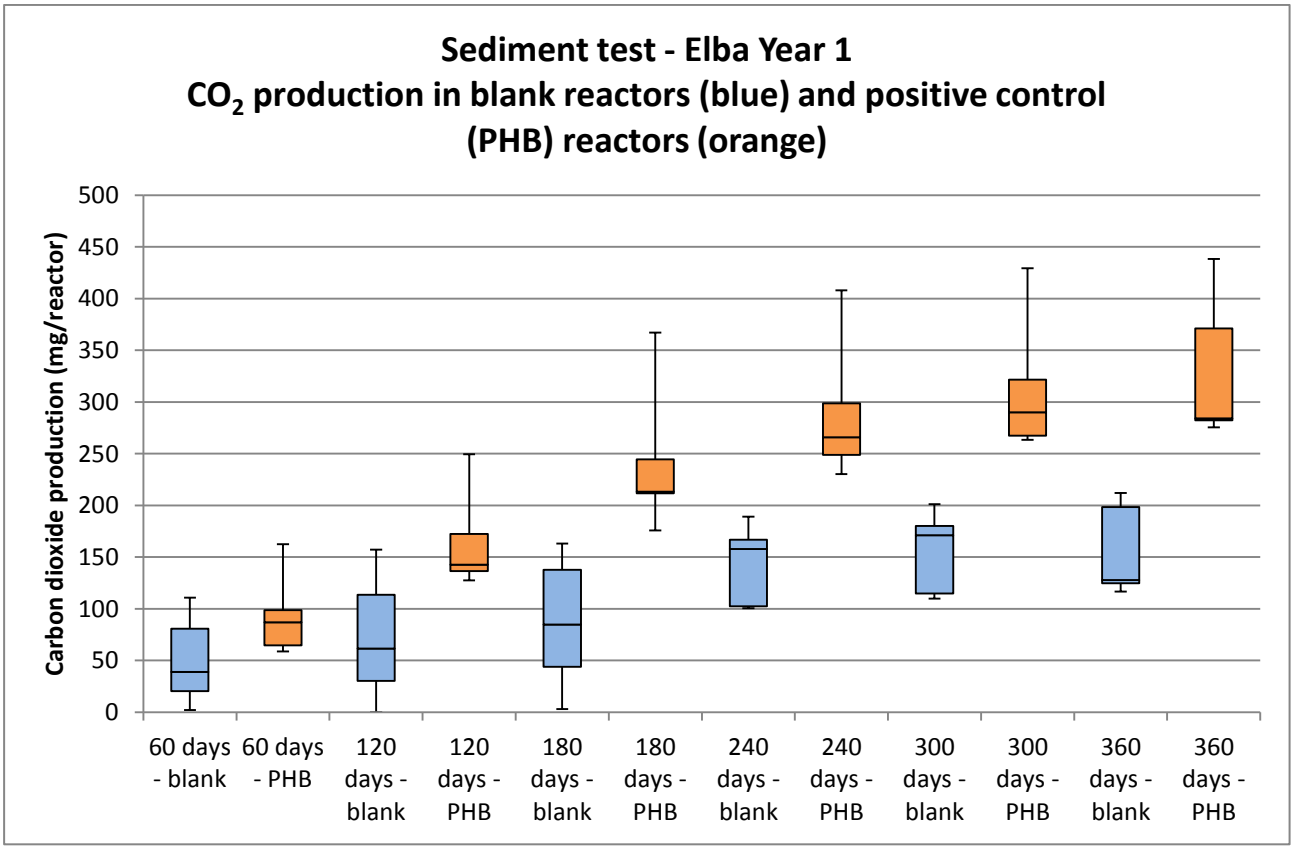


Figure 183. Results eulittoral (wet sediment) test - Elba Year 1; CO_2 production in blank reactors (blue) and positive control (PHB) reactors (orange)

7.3.2 Year 2 results

For the second year testing, the method was modified to increase the biodegradation speed. The nutrients addition was considered (basically N) as favourable modification. Also the addition of the test items in powder form was considered as a possible improvement and finally the possibility to bury the film test item in a reduced amount of wet sand in order to promote the diffusion of oxygen.

The second year results reported in Table 83 have been obtained following the test method with few modifications. The nutrient addition was tested (basically $NaNO_3$ at 0.1 g of N each g of test item organic carbon assuming a TOC of 50% for all samples) and in some cases also the test items were tested in powdered form. AUA explored also the increase of test item quantity combined with nutrient addition.

Comparing the biodegradation results between the two year tests is possible to note that a general increase of biodegradation rate was obtained in the second year. It is to remark the fact that several unrealistic biodegradation values (>100%) were obtained, while less the case in year 1. The increase of biodegradation rate could be due to the nutrient additions. However, it is difficult to state this with certainty since there remain unexplainable differences in sampling time, results from the different inocula and between the different laboratories.

Table 83. General summary of Eulittoral environment results year 2

Year 2 Eulittoral (wet sediment Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	240	134	224	257	
PHB	59.0 (±5.5)	64.1(±0.6)	50.5 (±19)	151.4 (±1.0)	-
PBSe	59.4 (±15.4)	41.5 (±1.3)	46.0 (±2.5)	86.7 (±18.3) *p 73.5 (±55.5) film	-
PBSeT	39.1 (±4.5)	50.2 (±5.3)	40.5 (±7.6)	69.1 (±12.4)	-
LDPE	-4.9 (±2.6)	1.8 (±0.2)	2.74 (±0.6)	3.4 (±0.8)	-
Year 2 Eulittoral (wet sediment Elba) **Biodegradation %**					
Test duration (days)	313			248	210
PHB	84.8 (±59.1)	-	-	99.4 (±12.3) *p 77.7 (±36.8) film	75.5 (±5.9)
PBSe	54.4 (±25.5)	-	-	88.9 (±5.4) *p 37.4 (±8.9) film	64.8 (±3)
PBSeT	51.8 (±11.1)	-	-	54.8 (5.9)	44.2 (±4.8)
LDPE	4 (±4.0)	-	-	-2.3 (±6)	-0.5

*p: powder

Other results were:

- Some laboratories carried out a parallel experiment using test items (PHB and PBSe) added in powder form. One laboratory run three tests (one using the Salamis sediment with PBSe test material and others two using the Elba sediment with PBSe and PHB test materials) in order to better compare the interaction between the nutrient addition with the use of powdered samples. The use of powdered sample poses an impossibility to verify the test material disintegration while the

biodegradation takes place. For this reason the powdered test materials were run in parallel with the same inoculum and the same test conditions with the reactors containing the test items in form of film. Results obtained using the powder are characterized by a higher biodegradation level joined to a lower standard deviation.

- LeAF, as during the year 1, performed the test at 20°C, obtaining lower level of biodegradation if compared to the other partners results (as expected as the other tests were performed at 28°C). Despite that, the standard deviation are generally lower when compared to the test performed at higher temperature (except PHB).
- BASF results highlighted a general improvement in test standard deviations as a result of the use of the Oxytop method (measuring the O_2 depletion instead of CO_2 production suggesting that this could be a valid approach).
- AUA highlighted the possibility to reduce the time of analysis and obtain well defined results (characterized by a low standard deviation) by combining a higher amount of test item with the addition of nutrients. A detailed description of these test method modifications are reported in the respective results section.

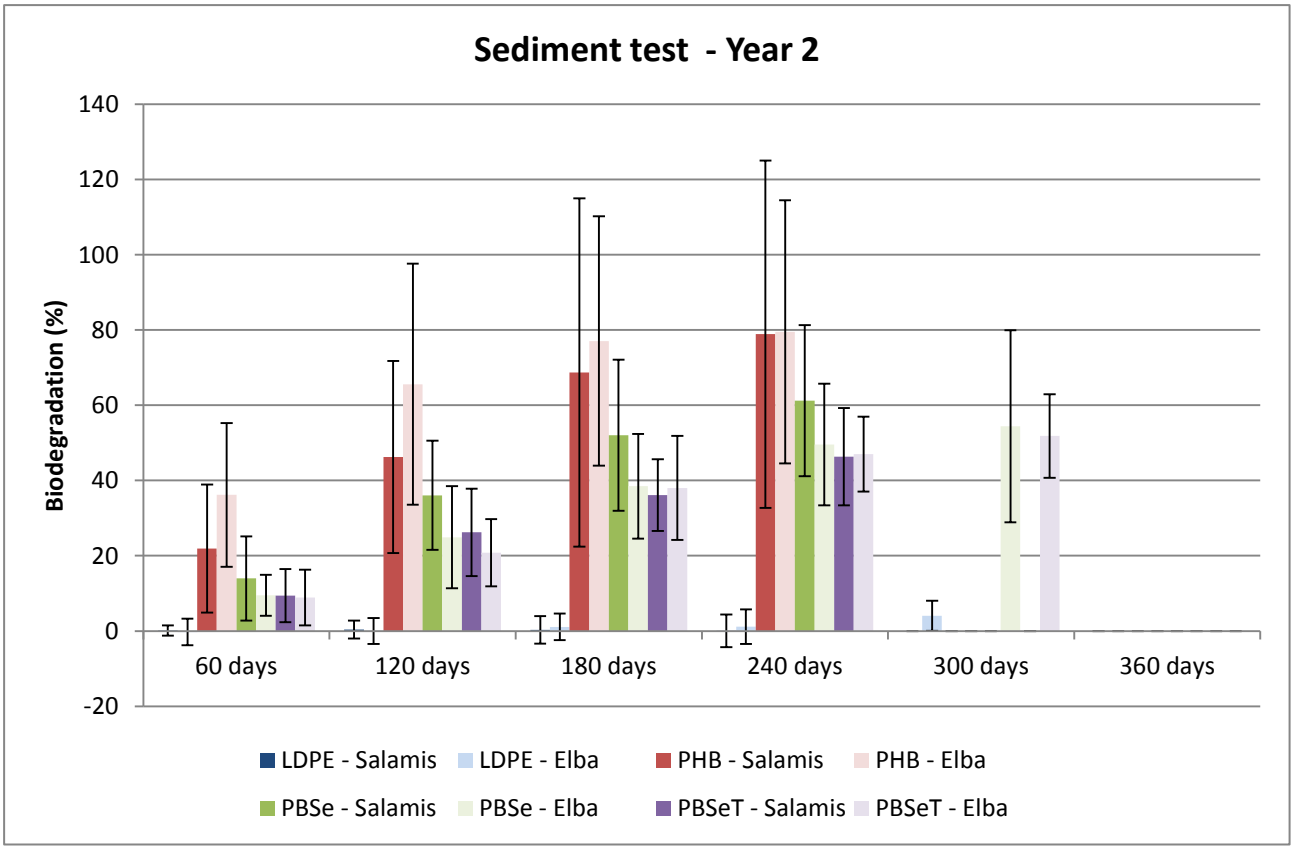


Figure 184. Results eulittoral (wet sediment) test - year 2; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

Taken together, the results (Figure 184) permit to do following observations regarding the modification applied to test method:

- Clear and well defined increasing biodegradation trend was confirmed for all materials (obviously except LDPE) and from both inocula during the test duration Differences in lag phases among triplicate bottles of the same test set.
- The test time required for (complete) biodegradation decreases but not significantly. Longer test times imply larger standard deviations in biological tests, because typically variations in microbial composition start to develop.

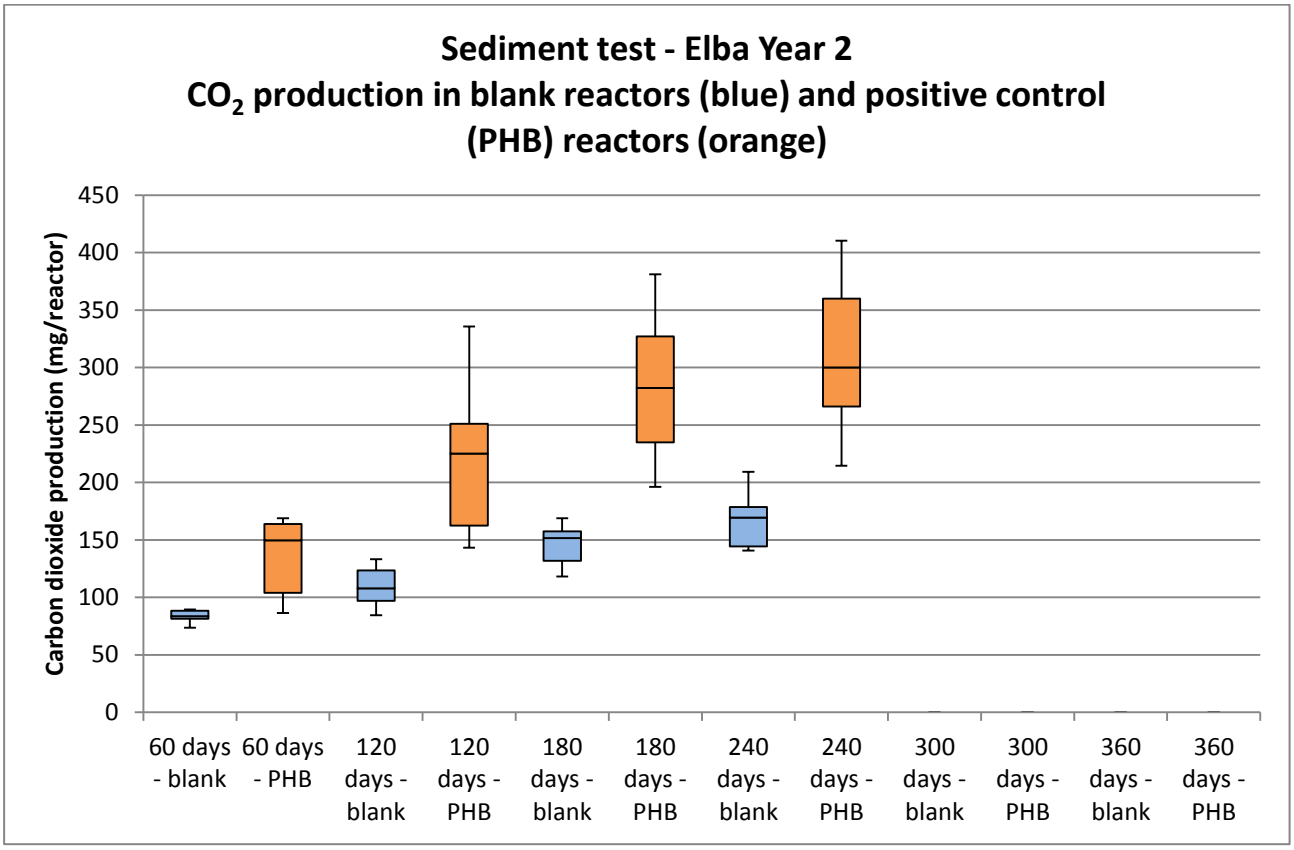


Figure 185. Results eulittoral (wet sediment) test - Elba Year 2; CO_2 production in blank reactors (blue) and positive control (PHB) reactors (orange)

Data concerning the CO_2 production (Elba sediment, year 2) were reported graphically in Figure 185. The graph shows that the overlapping (between the endogenous CO_2 with the PHB test item CO_2 at day 60) that was present during the first year at the beginning of the test tends to disappear. As the biodegradation of PHB started, the CO_2 production passed the endogenous ones and this separation remained stable for the entire test duration. At the end of the test the PHB test material produces two times more CO_2 than the endogenous. In general the “net” CO_2 production remains rather low and the increase of test material amount seems an advisable suggestion.

7.3.3 Conclusion

The test method used to perform the Eulittoral biodegradation determination seems to be promising due to well defined biodegradation trends that were obtained in year 1 and year 2 for both inocula. No overproduction of CO_2 was observed (only in one case with PHB test material and once with PBSe) and also a consequence, no LDPE biodegradation was registered.

The modifications adopted in order to decrease the length of the test time, which was generally recognized as the main problem of this method, were applied by the partners. Results indicated that:

- The nutrient addition (basically N) seems be correlated to a reduction of test duration.
- The use of powdered test items seems to be promising in order to increase the biodegradation rate at the early stage of the tests. This was tested only in one laboratory the suggestion is to confirm these results with further tests.
- Furthermore the use of higher quantity of test sample seems to be also a way to obtain well defined results in a relatively short period of time.

Concluding it can be stated that further experiments should be done, following the suggestion reported in this chapter, in order to complete the refinement process. The test method appears promising and could be suitable to future standardisation and certification procedures.

7.4 Test method: Pelagic environment – biodegradation in seawater

7.4.1 Year 1 results

In Table 84 the biodegradation results concerning the pelagic environment were reported. The test method adopted to perform the pelagic test was based on an existing American standard test methodology: ASTM D6691 *Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Sea Water Inoculum.* One deviation was made from ASTM D6691-09 related to the nutrient content (0.05 g NH_4Cl per liter seawater instead of 0.5 g/L NH_4Cl). In this specific set-up half of the involved laboratories performed the test using the Oxytop® device, measuring the O_2 consumption by a decrease of pressure. The use of this device permitted the double check by titration of the CO_2 absorber contained into the device (normally a KOH or NaOH solution). The others laboratories measured the CO_2 production by titration. Test items were added in powder form. AUA explored the possibility to fertilize the natural seawater with another fertilizer combined with a higher amount of test item.

Table 84. General summary of Pelagic environment results year 1

Year 1 Pelagic (free seawater from Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	312	84		180	
PHB	99.5 (±36.9) Replicates: 142% 75.4% 81.2%	70.7 (±0.8)	-	71.23 (±13.9)	-
PBSe	66.5 (±8.1)	66.3 (±0.9)	-	75.2 (±10.4)	-
PBSeT	73.1 (±9.8)	-1.7 (±0.5)	-	-1.2 (±4.4)	-
LDPE	-3.9 (±8.3)	-	-	2.3 (±1.2)	-
Year 1 Pelagic (free seawater from Elba) **Biodegradation %**					
Test duration (days)	312				200
PHB	80.2 (±47.5) Replicates: 44.6% 134.1% 62.0%	-	-	-	75.2 (±8.5)
PBSe	57.6 (±7)	-	-	-	26.0 (±11.6)
PBSeT	47.9 (±21.3) Replicates: 38.2% 72.3% 33.2%	-	-	-	52.9 (±46.9) Replicates: -1.2% 81.7% 78.2%
LDPE	-2.2 (±10.5)	-	-	-	2.1 (±1.2)

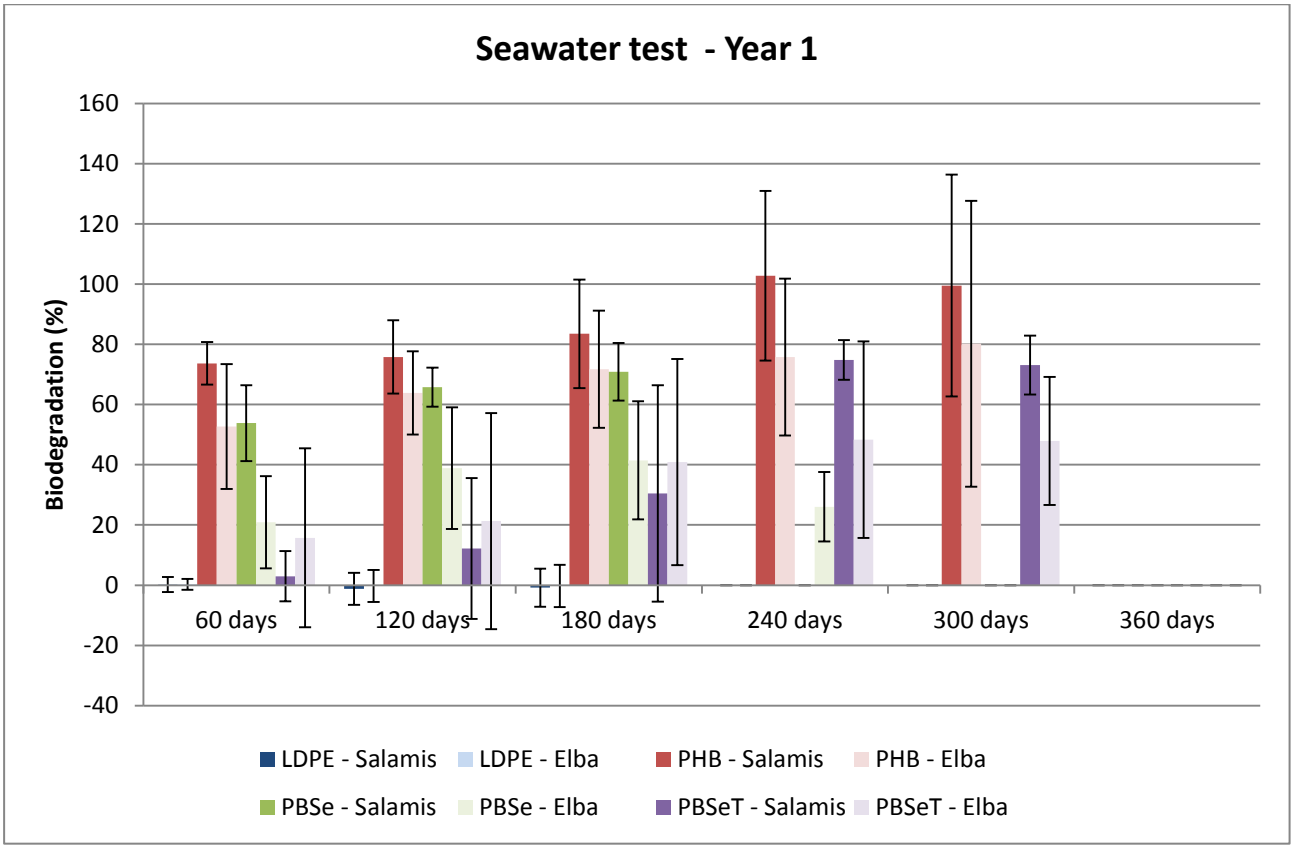


Figure 186. Results pelagic test - year 1; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

The pelagic experimental procedure seems to be more robust than the other tests as the results obtained (if we not consider temporarily the polyesters test materials) were in line between the laboratories. Taken together, the results permit to do the following observations regarding the test method applied:

- The CO_2 production in the LDPE series was comparable to the blank series, which is in line with the expectations.
- Differences between the two inocula were observed only for PBSeT
- Differences in lag phases among triplicate bottles of the same test set were observed only for PBSeT.
- Rather high standard deviations were observed
- Twice an overproduction of CO_2 was observed in one of the PHB reactors. This was observed for tests with a long duration. The deviation started after the plateau phase had been reached.

Results reported in Table 84 and Figure 186 highlighted that the biodegradation registered by the different laboratories is in line for PHB and PBSe samples. Concerning the PBSeT biodegradation trend, some differences were recorded. In detail, with the Elba inoculum two laboratories were not able to biodegrade the polyester while one lab obtained a

clear biodegradation of 73% with an acceptable standard deviation. With the Salamis inoculum both laboratories that carried out the test were able to biodegrade the PBSeT but clear differences between the replicates were observed. This behaviour was also previously noted during the biodegradation test in freshwater. It is possible that the chemical structure of the biodegradable polyester is biodegradable in seawater but the biodegradation only starts when a microbial consortium became well established for the presence of particular conditions in the reactor: stirring, temperature, nutrients, etc. Further investigations are necessary. No particular modifications were decided for the second year test.

7.4.2 Year 2 results

Table 85 shows the biodegradation results in the pelagic environment of year 2.

Table 85. General summary of Pelagic environment results year 2

Year 2 Pelagic (free seawater from Salamis) **Biodegradation %**					
	OWS	**AUA**	**LeAF**	**Novamont**	**BASF**
Test duration (days)	180	120		180	
PHB	74.7 (±1.4)	97.2 (±5.7)	-	68.7 (±6.3)	-
PBSe	78.2 (±6.8)	103.7 (±17.3)	-	64 (±2.7)	-
PBSeT	30.1 (±38.5) Replicates: -3.5% 72.1% 21.7%	78.2 (±46) Replicates: 45.7% 110.7%	-	18 (±24.5) Replicates: 46.3% 4.6% 3.1%	-
LDPE	-2.7 (±1.4)	12.4	-	-1.3 (±7.1)	-
Year 2 Pelagic (free seawater from Elba) **Biodegradation %**					
Test duration (days)	180			175	220
PHB	72.4 (±2)	-	-	84.7 (±3.1)	84.3 (±1.2)
PBSe	92.3 (±18.6)	-	-	31.8 (±37.1) Replicates: 74.5% 12.6% 8.3%	78.6 (±7.5)
PBSeT	78.4 (±13)	-	-	2.6 (0.8)	44.8 (±8.6)
LDPE	-0.7 (±3.3)	-	-	-0.9 (±0.3)	6.1 (±2.5)

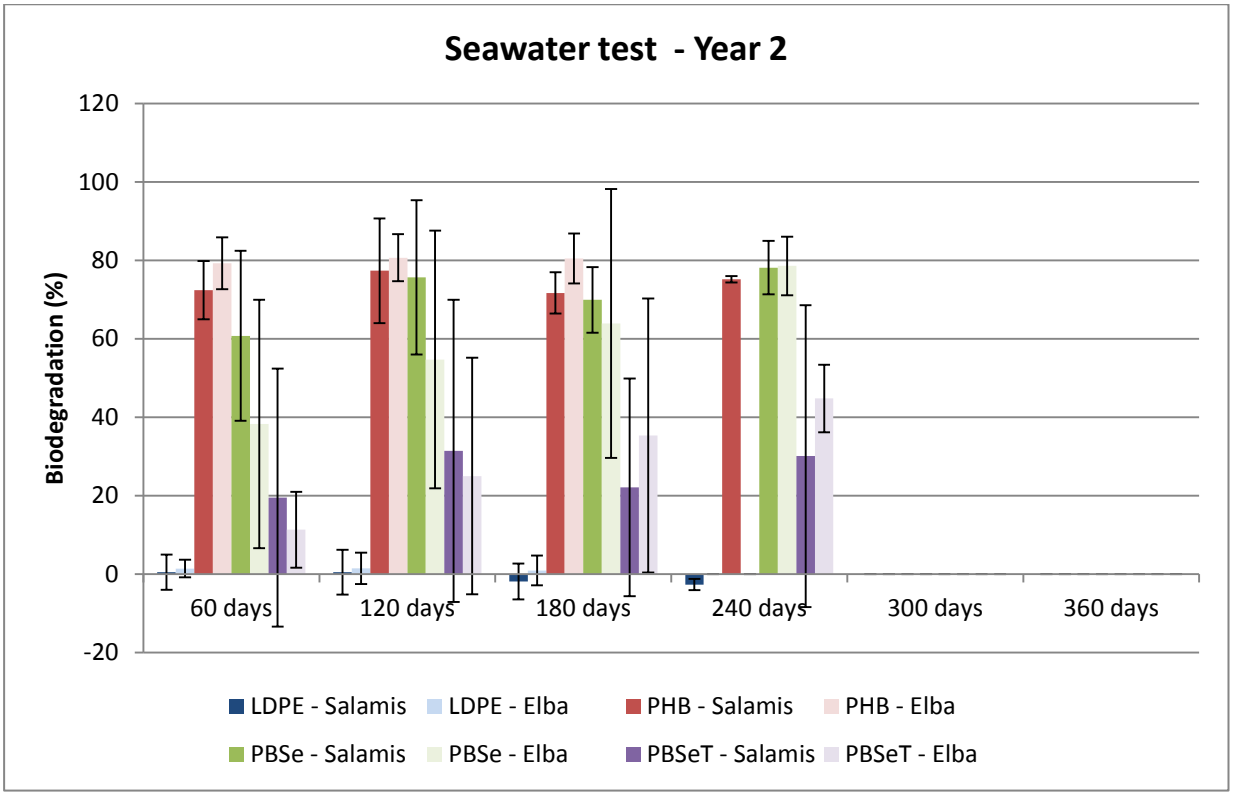


Figure 187. Results pelagic test - year 2; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

The second results confirmed the evidences of the first year:

- In general small deviations are observed between the different laboratories except for PBSeT
- The biodegradation results of PBSeT were, again, not reliable the reason remains unknown.
- A test duration of 120 days is necessary to complete the biodegradation
- It is possible to shorten the test duration by increasing the amount of sample but increasing also the fertilization
- Data concerning the CO_2 production (Elba seawater, year 2) were reported graphically in Figure 188. The graph highlighted a clear difference between CO_2 from blank reactors and CO_2 from PHB reactors, also the deviation are very small during the tests.

In some cases cellulose biodegradation in pelagic environment was tested as well resulting in very low biodegradation percentage. This behavior was unexpected because cellulose is generally used as a positive control in the standard biodegradation test methods. The reason could be that there is only a limited amount of microorganisms naturally present in the natural seawater that is capable to degrade cellulose.

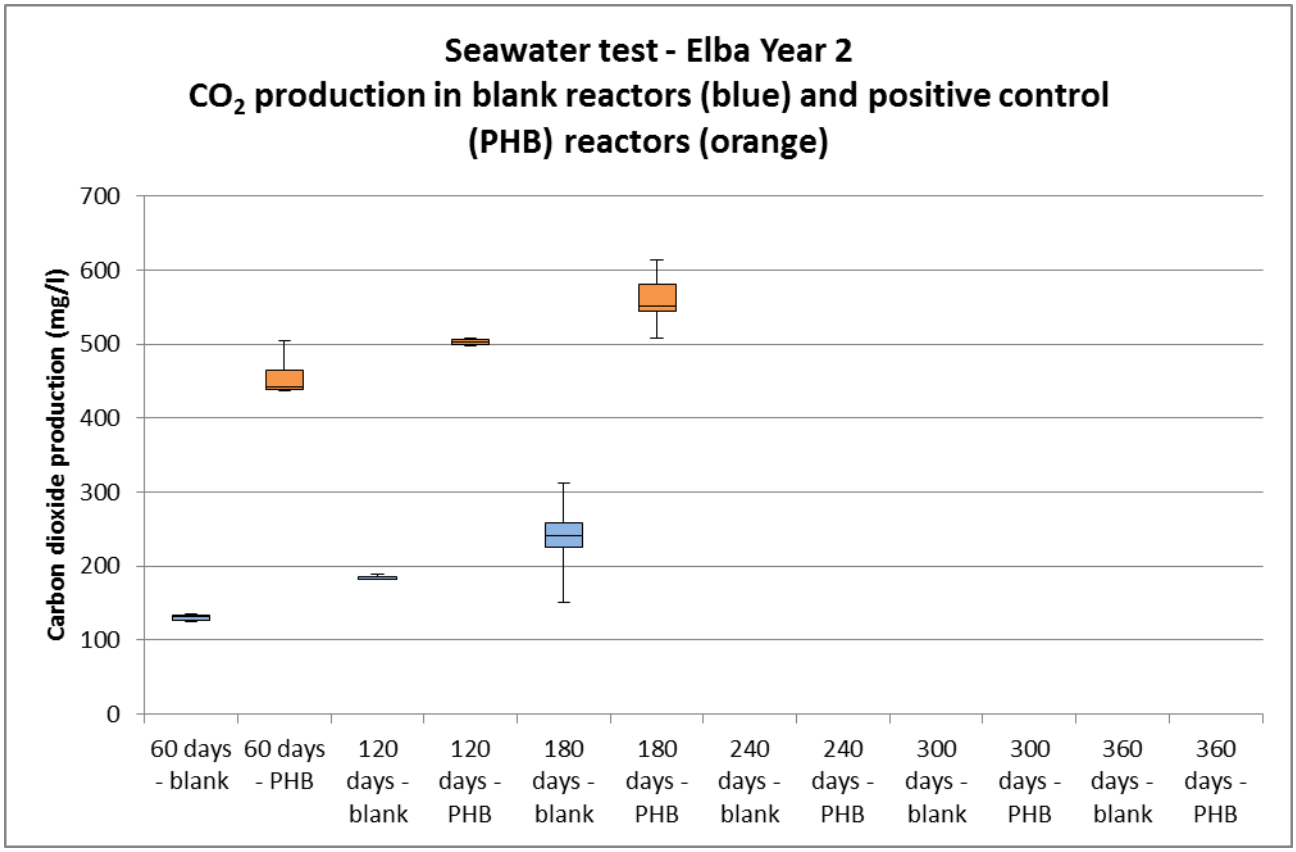


Figure 188. Results pelagic test - year 2; Biodegradation (%) of the test items in the different locations. Given are the average of the results and standard deviation.

7.4.3 Conclusion

The test method used to determine the biodegradation under Pelagic conditions seems to be promising as well defined biodegradation trends were obtained starting from year 1 and repeated in year 2 for both inocula. The test concentration of the sample guarantees a clear difference between the blank CO_2 production and the test material CO_2 production. Also the nutrients added seem sufficient. The test duration was around 120 days which is a time scale that can be considered acceptable. Results obtained with the aliphatic aromatic co polyester PBSeT were not reproducible. In the same experiment different biodegradation behaviour between the replicates was observed and during the two years some laboratories were able to biodegrade the test material and others not. The reason could be linked with the very low concentration of microorganisms in the seawater and/or the experimental conditions (temperature, stirring) that could influence the establishment of a particular microorganisms consortium. A good suggestion to increase the biodegradation of this kind of polyesters and avoid the problems reported previously, is the use of an aquarium bacteria inoculum or the addition of a small amount of sediment to increase the microbial concentration. Unfortunately no tests were performed yet using this approach.

Concluding it can be stated that further research investigation is needed to resolve the open questions (e.g. biodegradation of certain polyesters). If refined the test method appears promising and adapted to a future certification procedure.

8 References

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9 ANNEX A

Version no. 3
2015-07-10

Determination of aerobic biodegradation of bio-based materials buried in Sandy Marine Sediment -- Method by analysis of evolved carbon dioxide

MATERIALS

Reactor. Glass vessel approximately 2 to 4-L internal volume that can be sealed air-tight, such as 150-mm desiccators, with an airtight opening, large enough to allow the handling of the content. Biometer flasks are also appropriate.

Container for the CO_2 absorber. A glass beaker to be located in the headspace of the reactor and filled with 100 ml of $Ba(OH)_2$ 0.025 N or with 30 mL of KOH 0.5 N.

Darkened Chamber or Cabinet.

Analytical Balance.

Technical Balance.

pH Meter.

Barium Hydroxide Solution (0.025 N), prepared by dissolving 4.0 g anhydrous $Ba(OH)_2$/L of distilled water. Filter free of solid material and store sealed as a clear solution to prevent absorption of CO_2 from the air. It is recommended that 2 to 4 L be prepared at a time when running a series of tests. Confirm normality by titration with standard acid before use. When using $Ba(OH)_2$, however, care must be taken that a film of $BaCO_3$ does not form on the surface of the solution in the beaker, which would inhibit CO_2 diffusion into the absorbing medium. Alternatively, potassium hydroxide solution (KOH, 0.5 N) could be used and is prepared by dissolving 28 g of anhydrous KOH/L of distilled water and proceeding in the same way as for the $Ba(OH)_2$.

Hydrochloric acid. 0.05 N HCl when using 0.025 N $Ba(OH)_2$ or 0.3 N HCl when using 0.5 N KOH.

Sediment. Withdraw a sample of sandy sediment from the eulittoral zone of the shoreline, where the sediment is submerged by seawater at times other than low tide. It is preferable to obtain sediment from multiple locations. Collect sediment and seawater with a shovel into a bucket, then transfer the whole to a watertight container and quickly deliver it to the laboratory. Remove obvious plant material, sea shell, pieces of driftwood, petroleum tar, or other large pieces of material. Store the sediment and seawater at low temperature (approximately 4°C) until use. Use preferably within four weeks after sampling. Report the storage time. Before use, filter the sediment in a funnel with a coarse filter paper to eliminate excess water. Sediment is ready for testing when dripping of seawater is ended. Before to start the preliminary phase, to add at the sediment a source of Nitric Nitrogen (e.g. $NaNO_3$) an amount of 0.1 mg of N/ 1 mg of test material organic carbon should be used.

Note
It is advisable to prepare a batch of sediment with a sufficient amount to prepare all reactors (blanks , test materials and positive controls). For example if I foresee to add 400g of sediment in each reactor and I need of 10 reactors, I'll prepare at least 4000g of sediment. If I foresee to insert in the reactors, after preliminary phase, 100 mg of test material I can consider to add on average of 60mg of organic carbon so in each reactor I should add 6 mg of Nitrogen. This amount of nitrogen is calculated for 400mg of sediment (one reactor) and it will be multiplied for 10 in order to report to a total batch of sediment (4000g). This amount of nitrogen (60mg) is directly added to the batch sediment, dissolved in a little water, and mixed carefully. Then the inoculum will be added in the different reactors for the beginning of the preliminary phase.

Test material. Determine the total organic carbon (TOC) both of the test material and the reference material using e.g. ISO 8245 and report it, preferably, as grams of TOC per gram of total dry solids. Alternatively, provided the materials do not contain inorganic carbon, it is possible to determine the carbon content by elemental analysis. The test material shall have sufficient organic carbon to yield carbon dioxide in an amount suitable for the determination.

Reference material. PHB (Poly-β –hydroxybutyrate)

Test specimen. The material should be preferably in the form of film or plate. Cut out square-shaped specimens with a dimension of approximately 4-5 cm. Likewise prepare square-shaped specimens of reference material. Record the mass of each specimen.

PROCEDURE

Test set-up.
Prepare at least the following numbers of reactors :
a) 3 reactors for the test material;

b) 3 reactors for the reference material;
c) 3 reactors for negative control;
d) 3 reactors for the blank.

Preliminary phase.
Place between 200 and 600 g of sediment in the bottom of each reactor. In a typical case, weigh out 400 g of sediment and place it into the bottom of the reactor to form a homogenous layer. Do not press or compact the sediment. Introduce a container with the CO_2 trapping solution to each reactor. Close the reactors and locate them in a room or chamber kept at a temperature from 20 to 25°C, not exceeding 28°C. Monitor the CO_2 production.
This phase is carried out in order to verify that the endogenous respiration is similar in the different reactors and also to obtain a preliminary oxidation of excess organic matter, in order to start the test with a lower endogenous respiration.
This phase is generally protracted for a week. In case the CO_2 evolution of a reactor is different reject the diverging reactor or in case of multiple anomalies, start again using new sediment. It is advisable to mix the sediment during the preliminary phase in order to accelerate the degradation of organic matter.

Start of the test.
Open the vessels and remove about 100 g of sediment from the layer in the bottom of the reactor. Keep it in a clean container. Make smooth the surface of the residual sediment with a spatula but do not press it. Lay one or more specimen in order to reach 100 mg of test material or of reference material down on top of the residual sediment. No specimen is placed in the blank reactors. Replace the withdrawn sediment back in the reactor to form a homogenous layer that covers the specimens.

Carbon Dioxide Analysis
The carbon dioxide produced in each reactor reacts with $Ba(OH)_2$ and is precipitated as barium carbonate ($BaCO_3$). The amount of carbon dioxide produced is determined by titrating the remaining barium hydroxide with 0.05 N hydrochloric acid to a phenolphthalein end-point or by automatic titrator. Because of the static incubation, the barium carbonate builds up on the surface of the liquid and must be broken up periodically by shaking the container gently to ensure continued absorption of the evolved carbon dioxide. This problem can be avoided by using KOH instead of $Ba(OH)_2$, which does not form a precipitate.
The container for the CO_2 absorber must be removed and titrated before its capacity is exceeded. The period of time will vary with sediments and test materials and increases slowly as the carbon content of the sediment is reduced (a recommended frequency of once every week during the first month and every 2 to 3 weeks thereafter). At the time of removal of the containers, the reactor should be weighed to monitor moisture loss from the sediment and allowed to sit open so that the air in the reactor is refreshed before replacing 100 mL of fresh barium hydroxide and resealing the reactor. The reactors should remain open

approximately 15 min. Distilled or deionized water should be added back periodically to the sediment to maintain the initial weight of the reactor.
NOTE The minimum water content is the one that is retained by the sediment after filtration. The initial mass (wet sediment) shall be kept constant adding water.
Maximum test duration is 2 years.

CALCULATION

Amount of CO_2 produced. The first step in calculating the amount of CO_2 produced is to correct the test material reactors for endogenous CO_2 production. The control reactor serves as a blank to correct for CO_2 which may be produced through endogenous respiration of the microorganisms. The amount of CO_2 produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank containers. The next step is to convert ml HCl titrated into mg of CO_2 produced.

$Ba(OH)_2$ used as CO_2 absorber.
When CO_2 enters the absorber containers, it reacts in the following manner:
$Ba(OH)_2 + CO_2 \rightarrow BaCO_3\downarrow + H_2O$ (1)
The $BaCO_3$ formed is insoluble and precipitates. The amount of $Ba(OH)_2$ remaining in solution is determined by titration of the 100 ml with HCl according to the following equation:
$Ba(OH)_2 + 2\ HCl \rightarrow BaCl_2 + 2H_2O$ (2)
From the above two equations, it can be seen that 1 mmol of CO_2 is produced for every 2 mmol of HCl titrated. This means that the number of mmol of CO_2 produced:

$$\text{mmol } CO_2 = \frac{\text{mmol HCl}}{2}$$

The normality of HCl used is 0.05 N. Substituting for mmol gives:

$$\text{mmol } CO_2 = \frac{(0.05\text{ N}) \times (\text{ml of HCl})}{2}$$

To convert to mg CO_2, the value must be multiplied by the molecular weight of CO_2 which is 44:

$$\text{mg } CO_2 = \frac{((0.05) \times \text{ml titrated})}{2} \text{X } 44 = 1.1 \text{ ml of HCL titrated}$$

Thus, to convert ml of HCl to mg CO_2, the former is multiplied by 1.1.

KOH used as CO_2 absorber
The evolved CO_2 will react with KOH in the following manner:
$2KOH + CO_2 \rightarrow K_2CO_3 + H_2O$ (3)
K_2CO_3 , the product of reaction (3) is soluble and does not precipitate.

The fresh KOH solution, where no CO_2 has been absorbed, can be titrated with HCl as:
$KOH + HCl \rightarrow KCl + H_2O$, at pH 7 (4)
The KOH solutions used as CO_2 absorbers will have both unreacted KOH and K_2CO_3 as per (3).
During titration both chemical species will react with HCl, as follows:
$KOH + HCl \rightarrow KCl + H_2O$, at pH 7 (5)
$K_2CO_3 + HCl \rightarrow KHCO_3 + KCl$, at pH 8.5 (6)
The pH shifts of reactions (4) and (5) are superimposed and cannot be distinguished. Only a single end point in the range of pH between 7 and 8, corresponding to the two reactions, can be identified by using a suitable indicator.
The adsorbed CO_2 can be determined by subtracting from the H^+ equivalents needed to neutralise the original KOH solution and the H^+ equivalents needed to neutralise the reactions (5) and (6). In practice:
mmol CO_2 = [ml HCl consumed (4) – ml HCl consumed in (5) + (6) end point] * N HCl
where N is the normality of the HCl solution.
If an endpoint titrator is available the mmol of CO_2 can be determined, without using an indicator, with a further reaction. A further addition of HCl makes HCl react with $KHCO_3$, produced with reaction (6):
$KHCO_3 + HCl \rightarrow H_2CO_3 + KCl$ at pH 4 (7)
The number of equivalent consumed in reaction (7), and therefore in reaction (6), corresponds to the K_2CO_3 produced during reaction (3) that in turn corresponds to the absorbed CO_2.
Consequently 1 mole of $KHCO_3$ corresponds to 1 mole of CO_2 reacted in reaction ((3):
mmol CO_2 = mmol HCl consumed in (7) end point
Therefore:
mmol CO_2 = ml HCl consumed in (7) * N HCl
where N is the normality of the HCl solution.
The amount of CO_2 expresses in milligrams is finally obtained as follows:
mg CO_2 = mmol CO_2 *44

PERCENTAGE OF BIODEGRADATION

The percentage of biodegradation is the ratio between the evolved CO_2 and theoretical CO_2 ($ThCO_2$). The $ThCO_2$ is:

$$ThCO_2 = \text{specimen (mg)} \times \text{TOC (\%)} \times \frac{44}{12}$$

Where:
TOC (%) is the TOC of the test material (or reference material) divided by 100
44 is the molecular weight of CO_2
12 is the molecular weight of C
Therefore:

$$\% \text{ Biodegradation} = \frac{\text{mg } CO_2 \text{ produced}}{ThCO_2} X\ 100$$

VALIDITY CRITERIA

The reference material is necessary in order to check the activity of the sediment. If, after 180 days, limited biodegradation (<60 %) is observed for the reference, the test must be regarded as invalid and should be repeated using fresh sediment.

REPORT

Report the following data and information:
Information on the sediment, including source, pH, ash content, TOC, C:N ratio, date of collection, storage conditions, handling, and potential acclimation to test material.
TOC of the test material and reference material.
Form of the test materials.
Cumulative average carbon dioxide evolution over time to plateau (or termination), reported and displayed graphically.
Residual weight of the test material, if determined.
Percent of theoretical aerobic biodegradation for each test material tested and the reference material.
Temperature range of the test.
pH of the sediment, initially and finally.

10 ANNEX B

Version no. 3
2015-07-10

Determination of aerobic biodegradation of bio-based materials in a seawater/sediment interface-- Method by analysis of evolved carbon dioxide

MATERIALS

INCUBATION

Incubation shall take place in the dark at a constant temperature, preferably between 15°C to 25°C, but not exceeding 28°C, to an accuracy of ± 2°C.

REAGENTS

Seawater/sediment

Take a sample of a sandy sediment and seawater with a shovel at the shoreline directly from below the water line into a bucket. The wet sediment together with seawater is transferred into sealed containers for transport and fast deliver it to the laboratory. After delivery conserve the sediment at low temperature (approximately 4°C) until use. The seawater/sediment sample should be preferably used within 4 weeks after sampling. Record storage time and conditions.
Measure the TOC, pH and nitrogen content of the sediment. The carbon content of sediment should be in the range 0.5-3%.

APPARATUS

Test flasks

Biometer flasks of the volume of about 250 ml are appropriate. The vessels shall be located in a constant-temperature room or in a thermostatic apparatus.

Container for the CO_2 absorber

A glass beaker to be located in the headspace of the reactor and filled with 10 ml of $Ba(OH)_2$ 0.025 N or with 3 mL of KOH 0.5 N.

Analytical balance

pH meter

PROCEDURE

Test material

Test material should possibly have the form of a film, or a sheet. Cut samples of test material in the shape of a disk. Disks shall have a radius lower than the glass flasks' radius so that the disks can be easily laid on the bottom of the glass flask.

The sample shall be of known mass and contain sufficient carbon to yield CO_2 that can be adequately measured by the system used.

Use a test material concentration of at least 100 mg per litre of seawater plus sediment. The mass of the samples should correspond to a ThOD of about 170 mg/l or a TOC of about 60 mg/l. The maximum mass of sample per flask is limited by the oxygen supply to the glass flask.

Calculate the TOC from the chemical formula or determine it by a suitable analytical technique (e.g. elemental analysis or measurement in accordance with ISO 8245) and calculate the $ThCO_2$.

Reference materials

Use PHB as a reference material. If possible the form and size should be comparable to that of the test material. As a negative control, use polyethylene in the same form as the test material.

Test set-up

Provide a number of flasks, so that the test includes at least the following:

a) Three flasks for the test material (symbol F_T);
b) Three flasks for the blank (symbol F_B);
c) Three flasks for reference material (symbol F_C);
d) Three flasks for negative control (symbol F_N).

NOTE Two flasks for test material, blank, reference material and negative control may be used instead of three.

Preliminary phase

This preliminary phase is made to obtain an oxidation of excess organic matter in order to start the test with a lower endogenous respiration. To improve the effectiveness of the preliminary phase, a batch of sediment could be treated with a constant air flow (about 30 L/H) for 7-15 days depending of the organic matter content. If possible, the sediment could be gently mixed using a shaker. Should be measured and recorded the volatile solids at the beginning and at the end of the aeration phase. At the end of the preliminary phase, the sediment is filtered in a funnel with a coarse filter paper to eliminate excess seawater. Sediment is ready for testing when dripping of seawater is ended. Sediment after filtering is named "wet sediment" hereafter.

Another possibility is to dilute the sediment with the same sediment after calcination at 550˚C (in order to eliminate the organic content). A concentration of 50% is advisable. Using this approach, after the dilution, the sediment is directly inserted in the reactor with seawater and CO_2 trap, for a preliminary phase of 7 days. At the end of this phase and before to insert the test materials the CO_2 evolution is measure in each reactor.

Start of the test

In a typical case, use a test flask with a volume of 250 ml. Laid down 30 g of the wet sediment on the bottom of the flask. Carefully pour 70 ml of natural or synthetic seawater. In table B1 the composition of synthetic seawater.

Table B1

Sodium Chloride (NaCl)	22 g
Magnesium Chloride ($MgCl_2$*$6H_2O$)	9,7 g
Sodium Solfate (Na_2SO_4)	3,7 g
Calcium Chloride ($CaCl_2$)	1g
Sodium hydrogen carbonate ($NaHCO_3$)	0,20g

Make up to 1000 ml with deionized water.

Add carbon dioxide absorber to the absorber compartments of the test flask in a typical case 3 ml of KOH 0.5N or 10 ml of $Ba(OH)_2$ 0.025N. Place the flasks in a constant-temperature environment and allow all vessels to reach the desired temperature.
Dunk the plastic film sample, cut as previously described, on the sediment of each vessel. Mass of samples (test and reference material) should be about 20 mg each when using a flasks with a volume of 250 ml (Figure_B1). In order to assure a homogeneous contact between sample and sediment, it is recommended to cover the sample with a suitable coverslip. The coverslips must be introduced also in blank vessels, for assuring similar conditions.
NOTE A suitable coverslip can be made using a common non-biodegradable vinyl-coated fiberglass mosquito net with a fiber diameter of about 280 µm and a 1.8 mm x 1.6 mm mesh.

Figure B1 — Test Flask

Repeat the procedure for the reference material and the material for the negative control to the respective flasks. Record mass of sediment, sample and volume of seawater introduced in each vessel.

Carbon dioxide measurement

The CO_2 reacts with $Ba(OH)_2$ and is precipitated as barium carbonate ($BaCO_3$). The amount of CO_2 produced is determined by titrating the remaining barium hydroxide with 0.05 N hydrochloric acid to a phenolphthalein end-point or by automatic titrator. Because of the static incubation, the barium carbonate builds up on the surface of the liquid and must be broken up periodically by shaking the container gently to ensure continued absorption of the evolved CO_2. This problem can be avoided by using KOH instead of $Ba(OH)_2$, which does not form a precipitate.

The containers for the CO_2 absorber must be removed and titrated before their capacity is exceeded. The period of time will vary with sediments and test materials and increases slowly as the carbon content of the sediment is reduced (a recommended frequency of once every week during the first month and every 2 to 3 weeks thereafter). At the time of removal of the containers, the reactor should be allowed to sit open so that the air is refreshed before replacing 10 mL of fresh barium hydroxide and resealing the reactor. The reactors should remain open approximately 15 min.

The carbon dioxide evolution rate may reach a plateau when all of the accessible carbon has been oxidized. The test may be terminated at this point or earlier, at the discretion of the user. If possible, the residual test material may be extracted from the sediment with an appropriate method and quantified (optional).

End of the test

When a constant level of CO_2 evolution is attained (plateau phase reached) and no further biodegradation is expected, the test is considered to be completed. The maximum test period is 24 months. In the case of long test durations, special attention must be paid to the technical system (e.g. tightness of the test vessels and connections).

CALCULATION AND EXPRESSION OF RESULTS

Calculation

Amount of CO_2 produced

The first step in calculating the amount of CO_2 produced is to correct the test material reactors for endogenous CO_2 production. The control reactor serves as a blank to correct for CO_2 which may be produced through endogenous respiration of the microorganisms. The amount of CO_2 produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank containers. The next step is to convert ml HCl titrated into mg of CO_2 produced.

$Ba(OH)_2$ used as CO_2 absorber

When CO_2 enters the absorber containers, it reacts in the following manner:

$Ba(OH)_2 + CO_2 \rightarrow BaCO_3 \downarrow + H_2O$

The $BaCO_3$ formed is insoluble and precipitates. The amount of $Ba(OH)_2$ remaining in solution is determined by titration of the 10 ml with HCl according to the following equation:

$Ba(OH)_2 + 2\ HCl \rightarrow BaCl_2 + 2H_2O$

From the above two equations, it can be seen that 1 mmol of CO_2 is produced for every 2 mmol of HCl titrated. This means that the number of mmol of CO_2 produced:

$$mmol\ CO_2 = \frac{mmol\ HCl}{2}$$

The normality of HCl used is 0.05 N. Substituting for mmol gives:

$$mmol\ CO_2 = \frac{(0.05\ N) \times (ml\ of\ HCl)}{2}$$

To convert to mg CO_2, the value must be multiplied by the molecular weight of CO_2 which is 44:

Thus, to convert ml of HCl to mg CO_2, the former is multiplied by 1.1.

$$mg\ CO_2 = \frac{((0.05) \times ml\ titrated)}{2} \times 44 = 1.1\ ml\ of\ HCl\ titrated$$

KOH used as CO_2 absorber

The evolved CO_2 will react with KOH in the following manner:

$2KOH + CO_2 \rightarrow K_2CO_3 + H_2O$ (1)

K_2CO_3, the product of reaction (1) is soluble and does not precipitate.

The fresh KOH solution, where no CO_2 has been absorbed, can be titrated with HCl as:

$KOH + HCl \rightarrow KCl + H_2O$, at pH 7 (2)

The KOH solutions used as CO_2 absorbers will have both unreacted KOH and K_2CO_3 as per (1).
During titration both chemical species will react with HCl, as follows:
$KOH + HCl \rightarrow KCl + H_2O$, at pH 7 (3)
$K_2CO_3 + HCl \rightarrow KHCO_3 + KCl$, at pH 8.5 (4)
The pH shifts of reactions (2) and (3) are superimposed and cannot be distinguished. Only a single end point in the range of pH between 7 and 8, corresponding to the two reactions, can be identified by using a suitable indicator.
The adsorbed CO_2 can be determined by subtracting from the H^+ equivalents needed to neutralise the original KOH solution and the H^+ equivalents needed to neutralise the reactions (3) and (4). In practice:
mmol CO2 = [ml HCl consumed (2) – ml HCl consumed in (3) + (4) end point] * N HCl
where N is the normality of the HCl solution.
If an endpoint titrator is available the mmol of CO_2 can be determined, without using an indicator, with a further reaction. A further addition of HCl makes HCl react with $KHCO_3$, produced with reaction (4):
$KHCO_3 + HCl \rightarrow H_2CO_3 + KCl$ at pH 4 (5)
The number of equivalent consumed in reaction (5), and therefore in reaction (4), corresponds to the K_2CO_3 produced during reaction (1) that in turn corresponds to the absorbed CO_2.
Consequently 1 mole of $KHCO_3$ corresponds to 1 mole of CO_2 reacted in reaction (1):
mmol CO_2 = mmol HCl consumed in (5) end point
Therefore:
mmol CO_2 = ml HCl consumed in (5) * N HCl
where N is the normality of the HCl solution.
The amount of CO_2 expressed in milligrams is finally obtained as follows:
mg CO_2 = mmol CO_2 *44

Percentage of biodegradation

The percentage of biodegradation is the ration between the evolved CO_2 and theoretical CO_2 ($ThCO_2$). The $ThCO_2$ is:

$$ThCO_2 = specimen\ (mg) \times TOC(\%) \times \frac{44}{12}$$

Where:
TOC (%) is the TOC of the plastic material (or reference material) divided by 100
44 is the molecular weight of CO_2
12 is the molecular weight of C
Therefore:

$$\%Biodegradation = \frac{mg\ CO_2\ produced}{ThCO_2} \times 100$$

Visual inspection

At the end of the test check the conditions of samples. If still present, samples can be retrieved for mass determination, and other analysis, and photographs.
Expression and interpretation of results
Compile a table of the CO_2 values measured and the percentages of biodegradation for each measurement interval and each test flask. For each vessel, plot an evolved CO_2 cumulative curve and a biodegradation curve in per cent as a function of time.
A curve of averages may be plotted.
The maximum level of biodegradation determined as the mean value of the plateau phase of the biodegradation curve or the highest value, e.g. when the curve decreases or, further on, slowly increases in the plateau phase, characterizes the degree of biodegradation of the test material.
The wettability and the shape of the test material may influence the result obtained, and hence the test procedure may be limited to comparing plastic materials of similar chemical structure.
Information on the toxicity of the test material may be useful in the interpretation of test results showing a low biodegradability.

VALIDITY OF RESULTS

The test is considered valid if:
a) the degree of biodegradation of the reference material (F_C) is > 60 % after 180 days;
b) the evolved CO_2 of the blank F_B at the end of the test does not exceed an upper limiting value obtained by experience;
c) in the flasks F_N (negative control), no significant amount of evolved CO_2 shall be observed.
If these criteria are not fulfilled, repeat the test using another sediment.

TEST REPORT

The test report shall contain at least the following information:

- the main test parameters, including test volume, test medium used, incubation temperature and final pH;
- the source and amount of the marine sediment used;
- the source and amount of the natural or synthetic seawater used;
- TOC of the test material and reference material;
- the analytical techniques used, including the principle of the respirometer ;
- all the test results obtained for the test and reference materials (in tabular and graphical form), including the evolved CO_2, the percentage biodegradation values;

- the duration of the lag phase, biodegradation phase and maximum level of degradation, as well as the total test duration;
- any other relevant data (e.g. result of the visual final inspection and analysis of final samples, if still retrievable; photos of the final samples).

Open-BIO

Opening bio-based markets via standards, labelling and procurement

Work package 5

In situ biodegradation

Deliverable N° 5.7 Part 2:

Marine degradation test assessment: Marine degradation test of bio-based materials at mesocosm scale assessed

Confidential

Version: 1

September 2016

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The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° KBBE/FP7EN/613677.

Table of content

1 Publishable summary

The goal of the second part of this task was to develop a stand-alone mesocosm test to assess the degradation of polymers under partially controlled marine conditions. A closed-circuit tank system which mimicked the same three shallow-water habitats as in the laboratory tests, namely eulittoral (intertidal beach scenario), pelagic (water column scenario) and benthic (sublittoral seafloor scenario, sediment-water interface) within a single system was developed. The mesocosms were placed in a climate chamber where light, temperature, water movement, tides and water quality could be controlled complementary to laboratory tests. The volume of several hundred litres per habitat, and the use of natural sediment and seawater provided experimental conditions that were closer to the natural environment, and thus also allowed a link to field tests. Three independent mesocosm tank systems were run in parallel, two times consecutively, for the duration of one year each. The same polymers as in the laboratory tests (PHB, LDPE, PBSeT and PBSe) were tested.

The developed mesocosm system was well suited for the intended tests. Its simple construction and low technical effort proved to be reliable and efficient. Generally, all tested polymers, except the negative control LDPE, showed disintegration, with a differentiation by habitat and polymer type. However, there was a high variability in the rate of disintegration between replicates and between the experiments in year 1 and 2. Part of this heterogeneity could be explained by inhomogeneities in e.g. water movement, illumination and fouling, or slight differences in the system between the years, e.g. sediment grain size. Another part of the heterogeneity could not be explained *ad hoc*, and also be attributed to natural variations of the matrices water and sediment, and the microbial community therein. The observation of this high variability in a partially controlled test system and the analyses of the possible causes provided important information for the validation of laboratory and field tests.

The biodegradation of polymers, defined as the remineralisation to CO_2 (and/or CH_4) and water, and the conversion to biomass can only be directly measured in closed test systems where either CO_2 development or O_2 consumption is monitored, but not in the open tanks of the mesocosms. Therefore, material disintegration of polymer samples was estimated by the determination of lost area % over time. This technique provided a simple method to assess disintegration of polymer films, but had some intrinsic inaccuracies. The method was based on the visible lack of material and thus could only produce results once advanced disintegration had led to the perforation of the film. To assess the polymer degradation independently from eventual fragmentation there were also analytical methods applied to assess polymer disintegration on a macromolecular level like GPC and MALDI-TOF, but the results obtained did not show their suitability.

Methods that determined the mechanical properties of the tested materials at different exposure times gave satisfying results in case of slow degradation, but could no longer be applied for samples at an advanced stage of disintegration. Linked to the reliable determination of degradation is another question that could not be sufficiently addressed. Up to now no method could be applied that allowed to directly link the polymer biodegradation and specimen disintegration in the lab test to the disintegration of the same polymer in the mesocosm tests. Such a methodological link would be useful for a calibration of the tests in

the laboratory and in mesocosms, and furthermore also for field tests, and should be developed in further projects.

One disadvantage of the mesocosm tests performed was the relatively slow disintegration achieved at the applied temperature of 21 °C, which extended the necessary experiment duration to up to 1 year. Slight modifications of the conditions within a natural range, e.g. higher temperatures or the addition of nutrients could accelerate the disintegration and render the tests more practical.

The outcome of this part of the project is the proposal of a mesocosm test system suited to be applied independently from direct access to the sea with relatively low technical and financial effort. The mesocosm system can fill the gap of knowledge on the performance of biodegradable polymers under environmentally relevant marine conditions, in three of the most important coastal habitats. It can be developed into an additional test method to link the series of laboratory tests to field tests in the sea.

This ensemble of tests will open the possibility for materials and products to be tested under marine conditions in a reproducible environmentally relevant manner, and help society, producers and policy makers to verify claims of biodegradability.

Project website: www.Open-Bio.eu

2 Introduction

A mesocosm is an experimental system allowing to test environmental parameters under controlled conditions (Sala et al. 2000). It can be an enclosure system deployed directly in the natural environment or a tank system on land, ranging from 1 to >10000 L. A tank system can be run with flow-through seawater or with a closed water system. Within a tank system one tries to put the large ocean into a “small world” becoming able to fully control for example temperature, light and water movement conditions. It is possible to mimic the seasonal changes or to exclude them, for example to match part of the lab test conditions where constant temperature is applied.

Testing directly in the field under real environmental conditions would be preferable, but has major drawbacks and operational risks. In an open *in-situ* system it is possible to measure disintegration and chemical changes within the material but not biodegradation. Biodegradation is the remineralisation to CO_2 (and/or CH_4) and water, and the conversion to biomass by microbial action (ASTM D883 2000), and can only be tested in closed small-scale lab test systems, where the products can be quantitatively determined. Furthermore field tests are expensive because the systems need to be well constructed to withstand natural forces. Field test systems also have to be well located in order to minimise the risk of damage or loss by storms or by fishing activities. Due to limited access regular maintenance and sampling may require boats, heavy gear and specialised personnel. Performing field tests on an exemplarily level and applying combined standard mesocosm and lab tests on a regular level is therefore more feasible.

Mesocosm test systems have been used to examine the degradation of plastics (Stuparu et al. 2015) under controlled conditions more similar to nature than it is possible in small-scale laboratory tests. Thus mesocosm tests can serve as the bridge unit between field and lab tests and are currently applied within one standard test. In ASTM D7473 – 12 (ASTM D7473 2012) it says to “measure weight loss after samples have been exposed in a flow-through system”. This test still has limitations, and thus the further development of a mesocosm test system was the objective of this work within Open-BIO.

For the development of a mesocosm standard test HYDRA has established a closed mesocosm system in a climate chamber laboratory in order to allow its application independently from a direct access to the sea.

During the course of the Open-BIO project two consecutive experiments on the degradation of selected polymers, each running for one year were conducted. The chosen conditions were partly similar to the lab tests (Deliverable 5.7 part 1) performed in parallel and partly similar to the field tests (Deliverable 5.8). The tested polymers were analysed for disintegration, physical (tensile properties), and changes in molecular structure (GPC, MALDI-TOF). The temperature, light, salinity, oxygen content, nutrient and metal concentrations of the incubation media were regularly measured, and the data were used to evaluate the mesocosm system and test parameters for their suitability for a future standardisation activity.

3 Materials and methods

3.1 Test system and settings

3.1.1 General approach

The overall objective of the mescocosm experiments was to mimic, beyond laboratory test scale, the degradation of polymers in the marine environment in three coastal habitats: eulittoral, i.e. in an intertidal beach scenario, pelagic, i.e. in the free water column and benthic, i.e. on the seafloor. Two experiments were consecutively carried out each for the duration of approximately one year. HDPE plastic tanks (Dolav GmbH, Bad Salzuflen) with inner dimensions 93 x 113 x 60 cm were set up in triplicates in a climate chamber laboratory at 21 °C (Figure 189a). At each set the eulittoral test tank was placed on top of the tank with the benthic/pelagic test and connected by a closed-system water circuit of about 400 L seawater. Water was pumped into the upper tank in a way that a semidiurnal tide was mimicked, creating complete flooding every 12 h and a complete draining 6 h later. To follow the conditions during the experiments water and sediments from the tanks were sampled regularly and analysed in-house and by an external accredited analytical service (Institute Dr. Nowak GmbH & Co. KG, Ottersberg. Germany) for physical and biogeochemical properties with standard methods (for details see results section and Appendix table 1 up to Appendix table 9).

3.1.2 Seawater and sediment as incubation media

Natural Mediterranean seawater taken at Seccheto, Island of Elba, Italy was used to fill the mesocosms. Salinity was at 39. To re-adjust salinity for evaporation loss condensed water from the air dryer in the climate chamber and deionized water were used. Natural marine sediment for the eulittoral tests was retrieved at about 0.1 m water depth from the beach of Fetovaia, Island of Elba, Italy, and is subsequently called beach sediment. Sediment for the benthic tests was collected from the seafloor at 40 m depth off the Island of Pianosa, National Park Tuscan Archipelago, Italy, with the research permit n.3063/19.05.2014, and is subsequently called seafloor sediment. The sediment was brought to the laboratory and wet sieved with seawater through a 10 mm mesh in order to eliminate coarse particles as stones and shells, and was resuspended several times to flush out very fine particles. The physical sediment parameters grain size distribution, permeability and porosity were analysed with standard methods.

3.1.3 Environmental conditions

3.1.3.1 Temperature and light, salinity, pH and oxygenation

Temperature and light intensity were monitored with HOBO pendant temperature/light 64k data loggers (Onset Corp., Bourne, MA, USA). Light measurements were calibrated using a LiCor Underwater Quantum Sensor LI-192 (LICOR Inc., USA). Salinity, pH and oxygen concentrations were measured regularly in samples from the free water of the benthic/pelagic test tanks during both years' experiments, and from the sediment porewater in

the year 1 experiment at the level of the test material in the eulittoral test tanks with a conductivity sensor TetraCon® 925, a pH sensor SenTix® 940, and an oxygen sensor FDO® 925 attached to a Multi 3420 (WTW, Weilheim).

3.1.3.2 Water and sediment chemistry

In order to evaluate the water and sediment used in the mesocosm and to monitor the changes of its chemistry a selection of nutrient-related parameters (C, N, P, Si compounds), pigment and metals were analysed. Also toxic substances such as arsenic and heavy metals (cadmium, chromium, copper, lead, mercury, nickel, zinc), and a catalogue of known anthropogenic persistent organic pollutants were analysed in water and sediment from the tanks. Water samples from the benthic/pelagic test tanks were analysed from year 1 and year 2. Due to very low concentrations of some of the substances of interest the detection limits for some parameters were lowered by a modification of the method during the experiment. For the detailed list and the detection limits see Appendix table 1 to Appendix table 9.

3.1.4 Test material and preparation

Four polymer materials were selected to validate the disintegration in the mesocosm system: polyhydroxyalkanoate copolymer (PHB), polybutylene sebacate (PBSe), polybutylene sebacate co-butylene terephtalate (PBSeT), plus low-density polyethylene (LDPE) as the negative control Table 86.

Table 86. List of tested polymers with their properties supplier, film thickness and compounds. Percentage of total organic carbon (TOC), total carbon (TC), hydrogen (H) and nitrogen (N) were analysed with standard methods.

Test material	Note	TOC (%)	TC (%)	H (%)	N (%)
Low Density Polyethylene **LDPE** (negative control)	Grade: LUPOLEN 2420K Lyondelbasell Film 30 microns	85.03	85.37	14.68	< 0.1
Polybutylene Sebacate **PBSe**	Film 25 microns Aliphatic polyester	65.26	65.58	7.69	< 0.1
Polybutylene Sebacate-co-butylenterephtalate **PBSeT**	Film 25 microns Aliphatic-Aromatic polyester	65.25	65.81	9.54	< 0.1
Polyhydroxyalkanoate Copolymer **PHB** (positive control)	Film 100 microns, Grade: Mirel™ P5001 It is a compound > 70% PHB copolymer, plasticizer, fillers	47.82	49.11	6.03	0.52

Sheets of different polymer films were mounted in black plastic frames (PE-HD 300) of 260 x 200 mm external and 200 x 160 mm internal dimensions leaving a surface of 320 cm^2 exposed. The test material was covered on both sides with a semi-rigid white LDPE plastic mesh (General Cable, Highland Heights, KY, USA) with diamond-shaped meshes of 4 x 4 mm to prevent the eventual loss of fragments during disintegration. The percentage of

test material not shaded by the mesh was about 52 %. However, the mesh was not tightly adhering to the polymer film and thus allowed complete wetting of the sample.

3.1.5 Eulittoral tests

The eulittoral test tanks were filled with a layer of approximately 150 mm of beach sediment (Figure 189b). Sample frames were buried in the sand at 50-100 mm depth with an inclination of 11 ° from horizontal to prevent water to be trapped on the film at falling water level during the mimicked tidal cycles (Figure 189d). Twice a day the water level was risen above sediment level for 6 hours by pumping up seawater from the benthic/pelagic test tank below. When the pump was stopped the water was allowed to slowly drain through the bottom of the tank, with the consequence of sediment and samples falling dry. At the end of a test interval samples were carefully dug out of the sediment and processed as described below.

3.1.6 Benthic/Pelagic tests

The benthic/pelagic test tanks were filled with a layer of approximately 50 mm seafloor sediment. Sample frames for the benthic tests were placed onto the sediment surface and weighted with a block of natural granite rock to prevent it from being moved. The tank was filled with about 400 L of natural seawater as described above.

For the pelagic tests sample frames were hung from a rack perpendicularly in the water column (Figure 189c). The water was continuously moved by a water pump (EHEIM compact 300) with a pumping rate of about 300 L/h. The tank was illuminated from above in a 12:12 h rhythm by 2 fluorescent lamps BIOLUX L 36W/965 (OSRAM, Munich) with a nominal luminous flux of 2300 lm each. At the sediment surface of the tank light intensity was measured with a HOBO pendant temperature/light 64k data logger. The water volume was connected with the eulittoral test set on top of it, cycled by a pump (EHEIM compact 600) for 6 h twice a day. At the end of each test interval (see Table 87) polymer samples were retrieved from the tanks and processed as described below.

Figure 189. Mesocosm tank system. A) Overview of the mesocosm tank system. B) View into the upper tank, which mimicked the eulittoral (intertidal beach scenario) habitat. C) View into the lower tank, which mimicked the pelagic (water column scenario) and the benthic (sublittoral, seafloor scenario, sediment-water interface) habitat. The pelagic samples were hanging in the water and the benthic samples were placed on the sediment on the tank floor. D) View onto a sample, which was buried in the eulittoral sediment.

3.2 Sampling and analysis

3.2.1 Sampling intervals and sample preparation

In year 1 there were 3 polymers tested in the mesocosm experiments: low-density polyethylene (LDPE), polyhydroxyalkanoate copolymer (PHB) and polybutylene sebacate co-butylene terephtalate (PBSeT) and sampled at 4 time intervals (t_1 – t_4) (details see Table 87). In year 2 polybutylene sebacate (PBSe) was tested additionally and sampled at 2 time intervals (t_1 and t_3). Specimens of each test polymer were retrieved ca. every 2.5 months from the tanks. The last sampling interval of the second year experiment was only 1.5 months due to the termination of the project. The exact sampling dates are listed in Table 87**.** The polymer samples were photographed in their holding frame directly after the sampling, then removed from the frames and photographed again, then cut into subsamples. For an eventual later in-

depth investigation small subsamples were fixed for microscopy and molecular biology analyses with standard methods. The remaining part of the sample was rinsed in deionized water, air-dried and scanned in a flat bed scanner for the analysis of proportional material loss, stored at room temperature in plastic bags and sent to Novamont S.p.A. and ISA (Centre National de la Recherche Scientifique) for further analysis of tensile properties, and molecular composition by MALDI-TOF and GPC.

Table 87. Sampling dates of samples in the three habitats eulittoral (intertidal beach scenario), pelagic (water column scenario), and benthic (sublittoral, seafloor scenario) in the mesocosm test of the experiment of year 1 and year 2. *The last sampling interval of the second year experiment was only 1.5 months due to the termination of the project.

Eulittoral							
year 1				year 2			
interval	date	months	days	interval	date	months	days
t_0	22.09.2014	0		t_0	29.09.2015	0	
t_1	10.12.2014	2.5	79	t_1	15.12.2015	2.5	77
t_2	22.02.2015	5	153	t_2	28.02.2016	5	152
t_3	08.05.2015	7.5	228	t_3	24.05.2016	7.5	238
t_4	27.07.2015	10	308	t_4*	25.06.2016	9*	270
pelagic & benthic							
year 1				year 2			
interval	date	months	days	interval	date	months	days
t_0	22.09.2014	0		t_0	29.09.2015	0	
t_1	09.12.2014	2.5	78	t_1	15.12.2015	2.5	77
t_2	23.02.2015	5	154	t_2	27.02.2016	5	151
t_3	09.05.2015	7.5	229	t_3	23.05.2016	7.5	237
t_4	27.07.2015	10	308	t_4*	26.06.2016	9*	271

3.2.2 Disintegration area analysis

The disintegration (% area) was determined photogrammetrically: Dried samples were scanned on a LIDE 210 flat bed scanner (Canon Inc.) and analysed for the proportion of lost vs. still intact surface using the software ImageJ (https://imagej.nih.gov/ij/) and GIMP (http://www.gimp.org/). To allow numerical comparison of polymer films of different thickness the values were also normalised for thickness, obtaining a specific disintegration rate in volume per area and day as $cm^3\ cm^{-2}\ d^{-1}$.

3.2.3 Tensile properties, GPC, and MALDI-TOF analysis

Tensile properties

Tensile properties were determined in Novamont laboratories, following the ASTM D882 standard method. An Instron dynamometer (mod. 4301) was used. A speed of 50 mm/min was applied, the clamps had a distance of 50 mm. The samples were gently cleaned with distilled water and a piece of cotton, and then cut to the dimension of 10 x 150 mm. The

cutter machine was an ATSFAAR and the cutting procedure following as requested in ASTM D882. Before performing the tensile strength measurements, the samples were dried at laboratory conditions (23±1 °C, 50±5 % Rh) for not less than 48 h. All samples, which were still intact, and therefore able to be mounted into the dynamometer, were analysed.

GPC

Gel Permeation Chromatography was applied at Novamont laboratories to measure the molecular weight (Mn, Mw) and the polydispersity index (D) of a polymer sample. This analytical technique was chosen in order to follow in depth the macromolecular weight change of the polymer chemical structure while the biodegradation occurred. The molecules of a dissolved sample are differentiated in a chromatographic column packed with a porous gel according to their hydrodynamic volume (≅ "size"). We used an Agilent 1100 Series HPLC pump, equipped with two PL-gel columns (300 x 7.5 mm, 5 µm - mixed bed C and E) and a pre-column PL-gel guard (50 x 7.5 mm, 5 µm) connected in series, and an Agilent 1200 Series differential refractive index detector. Chloroform (HPLC grade) was used as eluent at a flow of 0.5 mL/min. The samples have been dissolved in chloroform at a concentration of about 1 g/L. The values for Mn, Mw and D were obtained on the basis of a universal calibration curve from polystyrene standards with the software Agilent GPC-Addon - Rev. B.01.01.
In order to understand if this technique was adequate for the purpose, the samples of PBSe and PBSeT were considered and tested. The polymers tested during the second year were analysed at time 0 and at the end of the test (PBSe: 9 months of exposure; PBSeT: 7.5 months of exposure). The chromatograms of the native and the most degraded polymers were compared for of each habitat.

MALDI-TOF

Matrix-Assisted Laser Desorption Ionisation - Time of Flight analysis allows to determine the repetitive unit mass of polymers and the total mass of end chains and was applied at the Centre National de la Recherche Scientifique. The sample is mixed with a matrix, ionised by a short laser pulse in a vacuum and accelerated by an electric field. After acceleration the molecules are separated according to the mass-to-charge ratio, which result in a differential time of flight to the mass detector. The polymer samples were dissolved in chloroform (10 mg/mL), centrifuged and diluted at a ratio of 1:10 with 2-(4-hydroxybenzeneazo) benzoic acid (HABA) at 10 mg/mL in tetrahydrofuran as matrix. The sample was analysed with an Ultraflex III SmartBeam TOF-TOF mass spectrometer (Bruker Daltonics, Bremen) in reflectron mode. The acquisition was performed in positive ionisation mode within a mass range between 1000 and 6000 Da. In order to obtain spectra for PBSe and PBSeT the laser power was increased when signal intensity of these polymers was very low. PBSe and PBSeT samples from experiment year 2, t_3 (238 days) from the eulittoral, the pelagic and the subittoral tests were analysed and compared to the analyses of untreated polymer samples. For methods details see Karas and Krüger (2003).

4 Results

4.1 Environmental data

4.1.1 Sedimentology

The beach sand used as matrix in the eulittoral tests was mainly of siliclastic origin and had the following properties (mean±standard deviation): in year 1 the mean grain size was 278±14 µm, the porosity was 0.45±0.02, and the mean permeability was 17.9±0.58 10^{-11} m^2, and in year 2 the mean grain size was 206±9 µm, the porosity was 0.42±0.08, and the mean permeability was 9.54±2.2·10^{-11} m^2 (Appendix table 5).

The sediment used for the benthic experiments was composed mainly of carbonate minerals and had the following properties: in year 1 the mean grain size was 181±21. the porosity was 0.62±0.04. and the permeability was 2.60±0.63·10^{-11} m^2, and in year 2 the mean grain size was 146±47, the porosity was 0.50±0.06, and the permeability was 2.4±1.2·10^{-11} m^2 (Appendix table 6).

4.1.2 Temperature and light, salinity, pH and oxygenation

Temperature varied around 20.5 °C by ± 1 °C (set: 21 °C) in all tanks and all experiments. Mean light intensity on the sediment surface of the benthic tests was around 385 lx, calibrated to 11.56 µmol photons/m^2·s.

Salinity, pH and oxygen content were measured regularly and the values were similar in all 6 tanks (3 replicates benthic/pelagic test and 3 replicates eulittoral tests). Salinity was about 39 with a variation of ±1 for both years (set: 38.5). The pH was stable at 8.1±0.1. The oxygen concentration was 7.1±0.2 mg/L and close to air saturation (98±2 %).

The high similarity of the replicate tanks within one one-year experiment and between the two consecutive experiments show the high reproducibility of the test systems in terms of general abiotic parameters.

4.1.3 Water chemistry

Nitrogen

The water in all tanks at the start of the experiments contained 1.2 mg/L (Appendix table 1 to Appendix table 3) total nitrogen $N_{tot.}$. N_{tot} decreased to two thirds to half the value in the three replicate systems in the first 2.5 months to about 0.6-0.9 mg/L respectively. That could be explained by a transformation into biomass and a loss to the system in the form of N_2 or other volatile N compounds. This is also reflected by the initial increase in dissolved total organic nitrogen to values about 0.6-0.7 after 2.5 months and a subsequent drop to about 0.3 or below after 308/270 days (t_4 year 1/t_4 year 2). The growth of bacteria and their release of extracellular polymeric substances might have been responsible for this. The concentrations of ammonia, nitrite and nitrate were close to or below the detection limit after 10 months. The constant decrease of available N compounds in the system points towards an increasing limitation of N during the course of the experiment.

Phosphor

The mean concentration of total phosphor in the water in the triplicate test tank systems were similar with a mean of 0.02-0.03 mg/L and thus generally low from the beginning of the experiment, with some variations from 0.01 (detection limit) to 0.11 mg/L (Appendix table 1 to Appendix table 3). Differential analysis of inorganic P (ortho-phosphate) showed very low values in all samples close to or only slightly above the detection limit (0.005 mg/L). Thus most of the total (dissolved) P in the water was organic P. According to these results the system did not seem to be P-limited after 308 days.

Carbon

The mean concentrations of total carbon (TC) in the water in the triplicate test systems were around 30-40 mg/L with some variation over the course of the experiments and between tanks (Appendix table 1 to Appendix table 3). There was no obvious trend in time. The mean concentrations of dissolved organic carbon (DOC) in the tanks of the pelagic/benthic tests were around 3 mg/L. In the porewater of the sediment of the eulittoral test tanks mean DOC concentrations were around 5 mg/L, with an increase to about 8-11 mg/L after 5 months of year 1, but no obvious trend over the duration of the whole experiment. The relatively stable values of DOC in all tanks showed that the biotic conditions in these small ecosystems were quite balanced and no accumulation or complete depletion has occurred. DOC production by e.g. algal exudates, cell lysis or other sources seemed to be kept in balance by microbial and algal growth within the system.

Chlorophyll a/Phaeopigments

The concentration of chlorophyll *a* (Chl *a*) is a measure for photosynthetically active protists (“microalgae“) and bacteria in the water (Appendix table 1 to Appendix table 3). The degradation products of Chl *a*, formed once the organisms are dead, are summarized as phaeopigments. Initially Chl *a* was below detection limit in the water from the freshly filled test tanks, phaeopigment concentration was low (1.5 µg/L). During the year 1 experiment the Chl *a* values in the pelagic/benthic test tanks were low (3.6-0.3 µg/L) or below detection limit. The small increase reflected the visible establishment of a phototrophic community of algae and bacteria at the tank and sediment surfaces in the first half of the experiment (150 days), which remained stable until the end of the experiment (308 days). In the sediment porewater the Chl *a* concentration was slightly higher and detectable in almost every sample. This could be explained by the filtering effect of the sediment which was percolated by the water from both tanks twice a day. Phaeopigments were above detection limit in most samples from the pelagic/benthic test tanks (mean 1.6-2.4 µg/L) and in all of the porewater samples from the eulittoral test tanks (mean 5.0-5.9 µg/L). The moderate pigment concentrations corroborate the observation that no bloom or mass development had occurred in the water phase of these closed tanks and that the test systems were ecologically quite stable. In the year 2 experiment pigments were not analysed.

Silica, Iron, Manganese and Aluminium

The concentration of silica in all test tanks was slightly above the detection limit

(0.4 mg/L) or not detectable over the course of the experiments (Appendix table 1 to Appendix table 3). The detection of silica in a few samples showed the presence of this micro-nutrient that in natural waters can be limiting for diatoms and some other skeleton-forming protists.

Iron was detected (> 0.1 mg/L) only once in one test tank. This could mean that there was no substantial mobilisation of iron minerals by solution due to regularly changing conditions of water saturation and aeration during the mimicked tides in the eulittoral test tanks.

Manganese was not detected in the water (detection limit 0.05 mg/L). Like iron, no manganese minerals of the sediment seemed to be dissolved in the porewater during the tidal cycle, or might mostly remain undetected due to low concentrations.

Aluminium concentrations were around 0.1 mg/L in almost all samples from the test tanks for year 1. In year 2 only the first sampling revealed aluminium concentrations of about 0.2 mg/L. In all other samples Al was below the detection limit of 0.05 mg/L. Aluminium also seemed not to be dissolved in the tidal cycle within the sediment porewater.

Generally, Si, Fe, and Mn serve as micro-nutrients to living organisms. The experimental design with the use of natural siliclastic sand that contained a variety of minerals together with an occasional detection of dissolved metal compounds in the water samples suggest that these micro-nutrients have been available to the organisms in the test tanks and have not been limiting factors.

Toxic substances: As, heavy metals, organotin compounds and persistent organic pollutants

Arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc, and a catalogue of known anthropogenic organic toxins were analysed in one water sample from the tanks. None of the substances was detected (Appendix table 4 and Appendix table 8).

4.1.4 Sediment chemistry

Nitrogen

Total N was below detection limit (1 g/kg) in both sediments (Appendix table 5 and Appendix table 6).

Phosphorous

Total P was around 200 mg/kg in the seafloor sediment (benthic test) and around 55 mg/kg in the beach sediment (eulittoral test) (Appendix table 5 and Appendix table 6).

Carbon

Total organic carbon (TOC) was low at 0.3 % dry weight in the seafloor sediment (benthic test) and below detection limit (0.2 % dw) in the beach sediment (eulittoral test) (Appendix table 5 and Appendix table 6).

Heavy metals and arsenic

Lead concentrations were 5-6 mg/kg in the seafloor sediment and below detection limit (d.l.) of 5 mg/kg in the beach sediment. For the detection limits see Appendix table 5 and

Appendix table 6. Nickel concentrations in the beach sediment were 9 mg/kg and below detection limit of 5 mg/kg in the seafloor sediment. Chromium concentrations in the seafloor sediment were 5-6 mg/kg and 30 mg/kg in the beach sediment. Cadmium (d.l. 0.5 mg/kg), zinc (d.l. 10 mg/kg), copper (d.l. 5 mg/kg), mercury (d.l. 0.05 mg/kg) and arsenic (d.l. 5 mg/kg) concentrations were below detection limits. Especially As, Cr and Pb but also Ni and Cd concentrations in the whole coastal region of Tuscany are known to be naturally elevated due to its geological setting. The measured concentrations in the sediments used for the experiments were below the environmental quality standard (EQS) values of the European Union (EU EQS for sediment: As 12 mg/kg dw, Cr_{tot} 50 mg/kg dw, Cd 0.3 mg/kg dw, Hg 0.3 mg/kg dw, Ni 30 mg/kg dw, Pb 30 mg/kg dw) (EU 2013) (ARPAT 2015).

Toxic substances: organotin compounds and persistent organic pollutants

None of the known anthropogenic organic toxins analysed in the sediments used for the experiments was detected. For the list of substances tested and the detection limits see (Appendix table 9).

4.2 Polymers

4.2.1 Visible disintegration and biofilm

Visible signs of disintegration were translucent areas due to progressive thinning of the polymer films (most prominent in PHB), the appearance of cracks, holes and finally fragmentation. Visible changes in material integrity were observed for PBSeT, PBSe and PHB in all habitats and both years (from Figure 190 to Figure 195). In some cases however the optical differences were in colour and thus difficult to tell apart from discolouration by biofilm growth and mineral precipitation. For LDPE no visible signs of disintegration could be observed.

All samples showed a change in colour during the time of the experiment. This can be attributed to the formation of biofilm and mineral precipitates. The eulittoral samples were slightly covered with a somewhat slimy opaque film (Figure 190 and Figure 191). A greenish-olive film developed on the pelagic samples (Figure 192, Figure 193 and Figure 196) and a film of green, reddish and rusty areas was found on the benthic samples (Figure 194, Figure 195 and Figure 197), where the polymer itself was mostly covered with microscopic organisms. Observations indicated a polymer-specific colouration which might reflect a microbial community specific for each polymer. Whether this also reflects microbes specifically involved in the degradation of the polymer remains to be further investigated. The protecting frame and mesh was also populated by filamentous algae and small invertebrate animal colonies (Figure 196 and Figure 197).

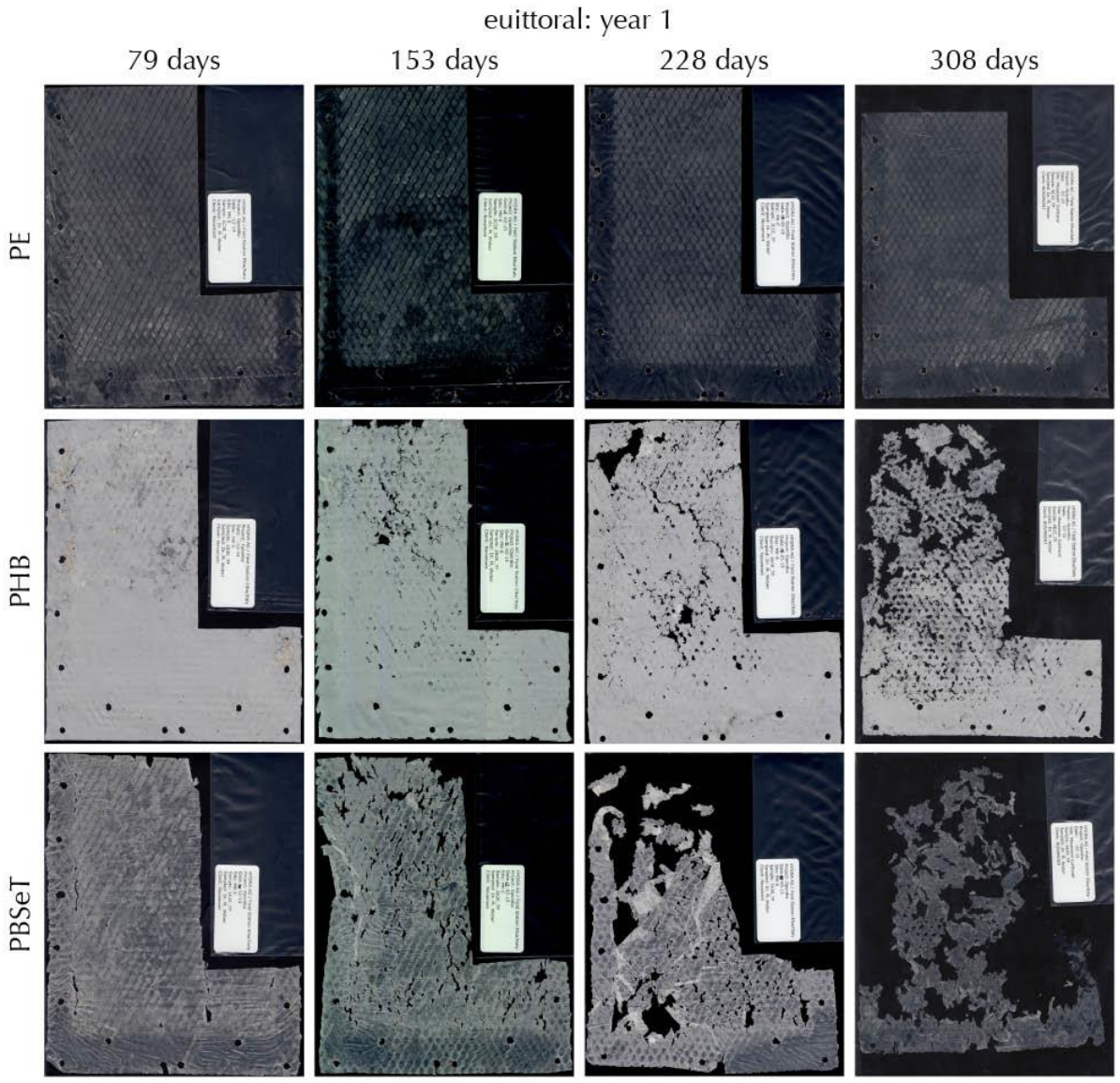


Figure 190. Exemplary scan images of LDPE, PHB and PBSeT samples from the mesocosm eulittoral (intertidal beach scenario) tests from year 1. PHB and PBSeT showed disintegration gradually progressing with exposure time. No disintegration was visible for LDPE, but the bio-film on the polymer was well discernible.

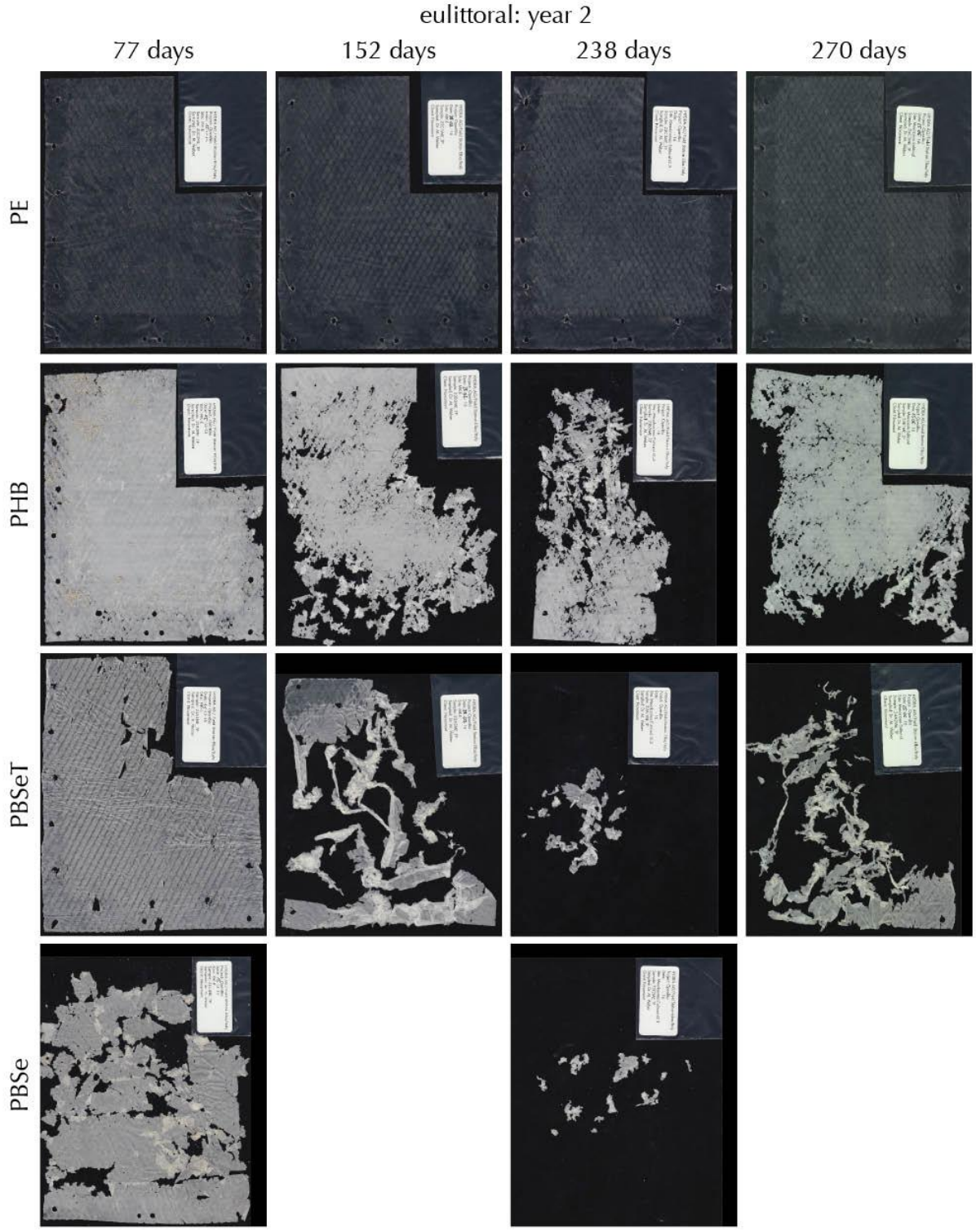


Figure 191. Exemplary scan images of LDPE, PHB and PBSeT samples from the mesocosm eulittoral (intertidal beach scenario) tests from year 2. PHB and PBSeT showed heterogeneous disintegration with exposure time. The heterogeneity is further assessed in the main text. PBSe samples were only exposed for 152 and 238 days. No disintegration was visible for LDPE, but the biofilm on the polymer was well discernible.

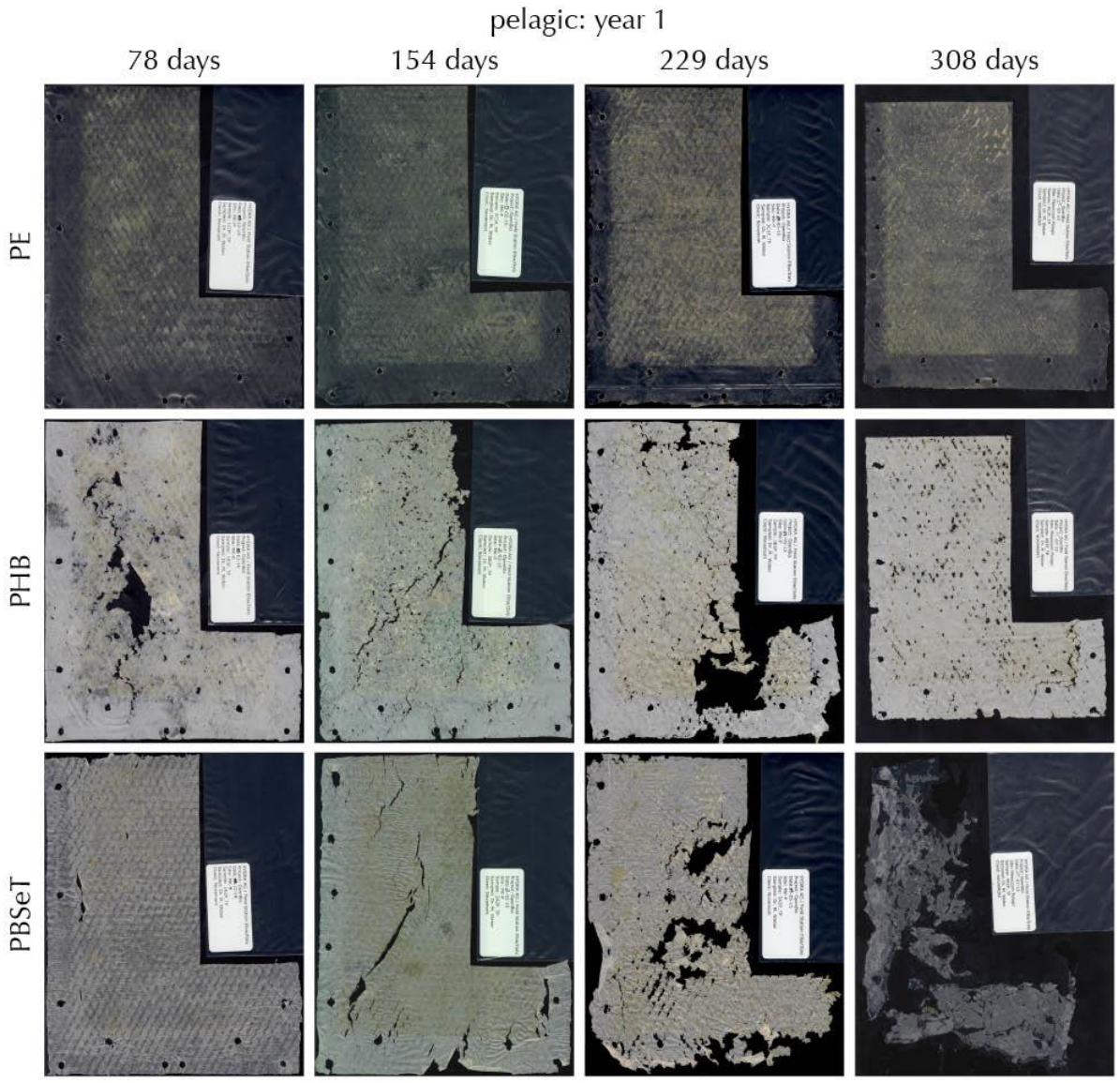


Figure 192. Exemplary scan images of LDPE, PHB and PBSeT samples from the mesocosm pelagic (water column scenario) tests from year 1. For PBSeT disintegration was gradually progressing with exposure time. For PHB disintegration was heterogeneous over time, further addressed in the main text. No disintegration was visible for LDPE, but the biofilm on the polymer was well discernible.

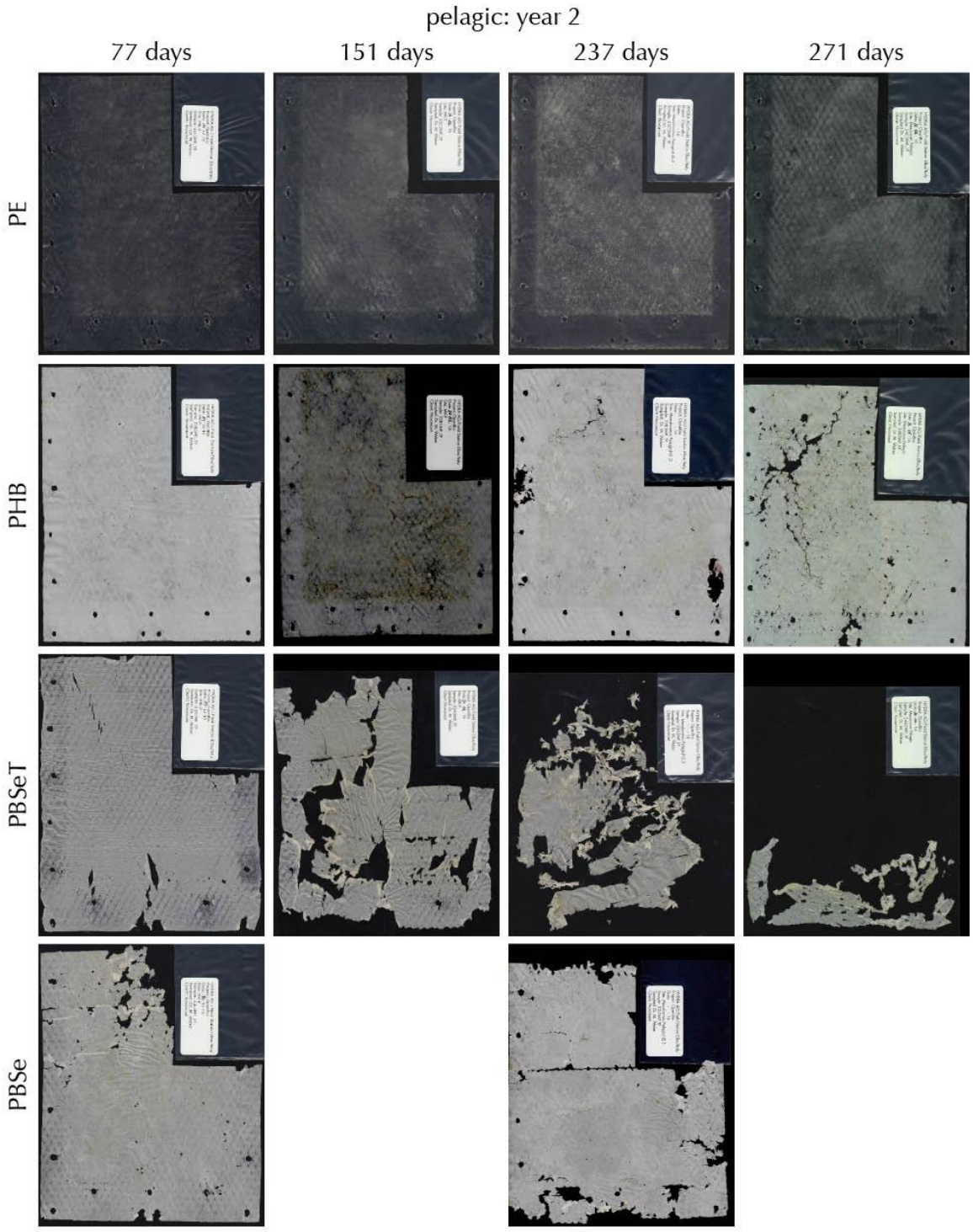


Figure 193. Exemplary scan images of LDPE, PHB, PBSeT and PBSe samples from the mesocosm pelagic (water column scenario) tests from year 2. For PBSeT disintegration was gradually progressing with exposure time. For PHB disintegration was heterogeneous over time, further addressed in the main text. PBSe samples were only exposed for 151 and 237 days. No disintegration was visible for LDPE, but the biofilm on the polymer was well discernible.

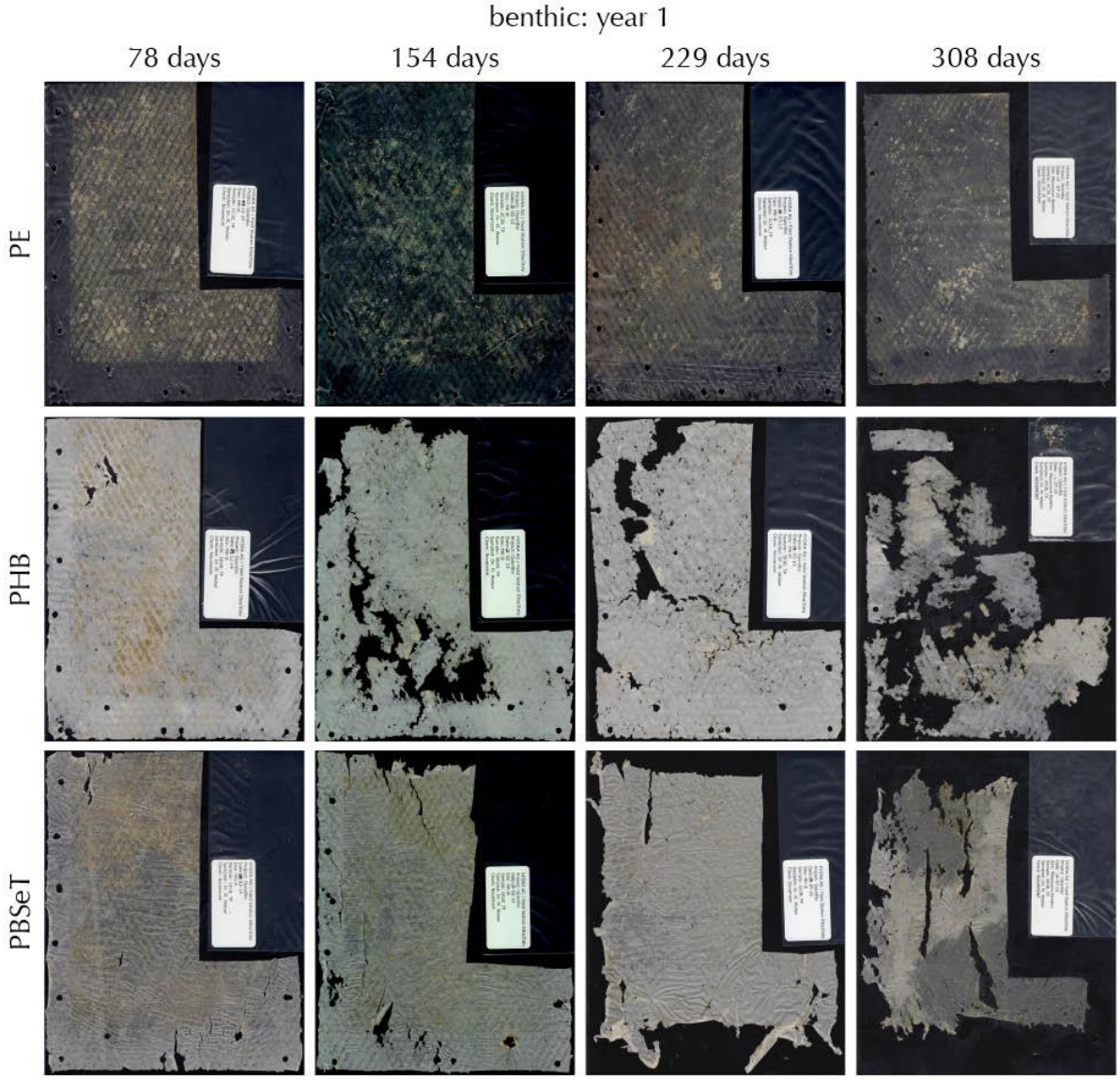


Figure 194**. Exemplary scan images of LDPE, PHB and PBSeT samples from the mesocosm benthic (seafloor scenario) tests from year 1. For PBSeT disintegration was gradually progressing with exposure time. For PHB disintegration was heterogeneous over time, further addressed in the main text. No disintegration was visible for LDPE, but the biofilm on the polymer was well discernible.**

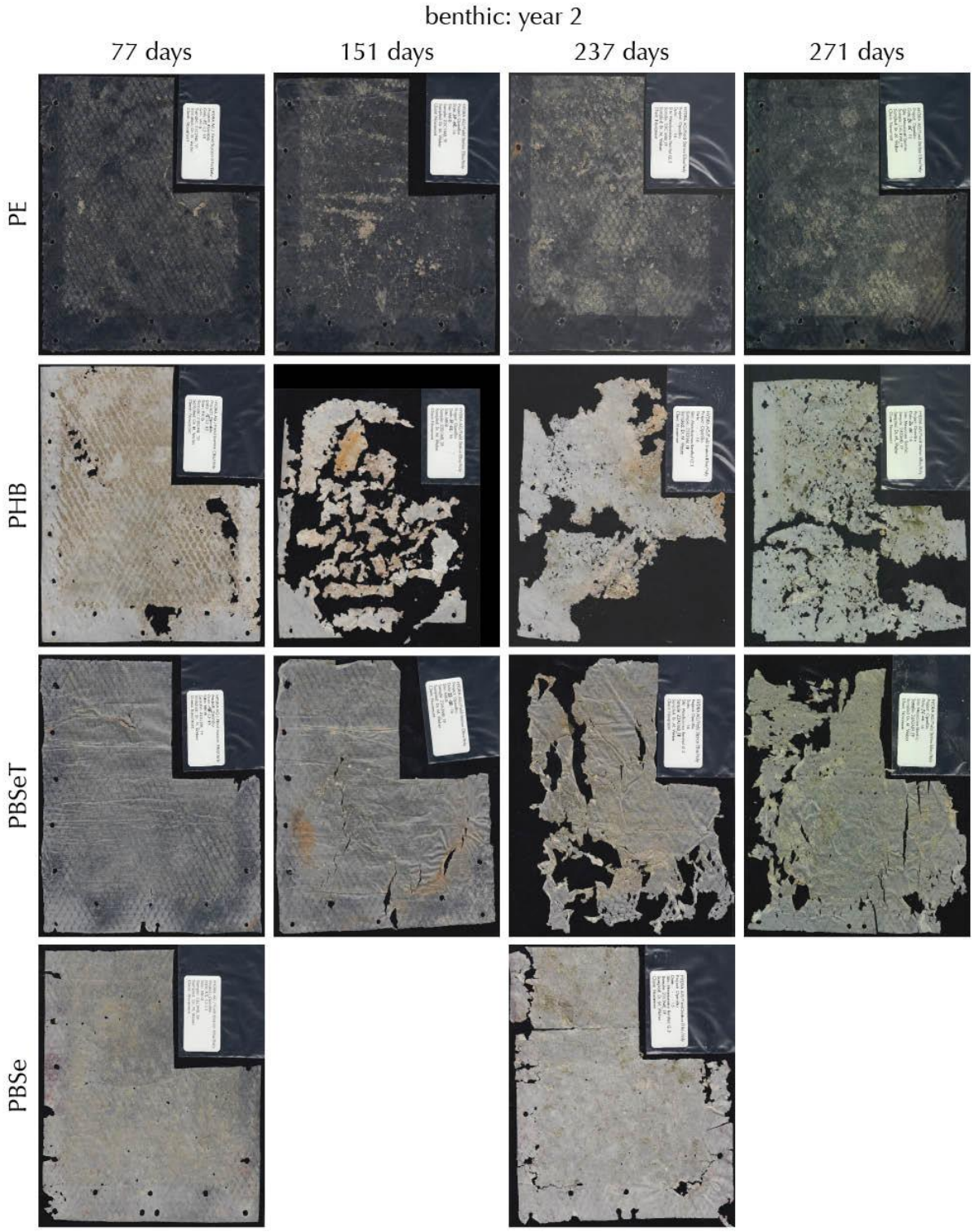


Figure 195. Exemplary scan images of LDPE, PHB, PBSeT and PBSe samples from the mesocosm benthic (seafloor scenario) tests from year 2. For PBSeT and PHB disintegration was heterogeneous over time, further addressed in the main text. PBSe samples were only exposed for 151 and 237 days. No disintegration was visible for LDPE, but the biofilm on the polymer was well discernible.

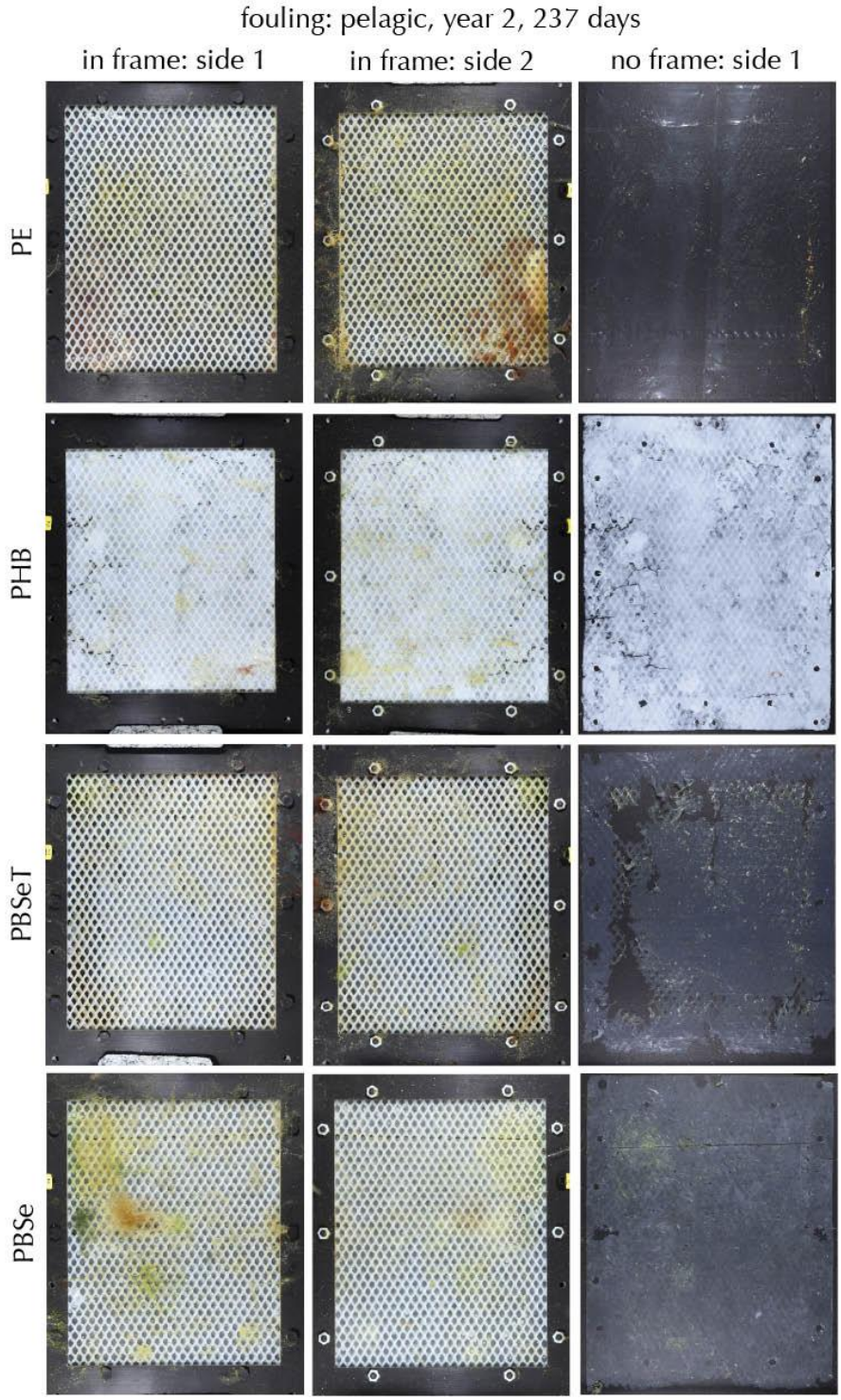


Figure 196. Exemplary images of LDPE, PHB, PBSeT and PBSe fresh samples from the mesocosm pelagic (water column scenario) tests from year 2 after 237 days of exposure showed the biofilm formation (fouling). Left and middle column are images of the polymer film still in the sample holder, right column shows the polymers after their removal from the frames. Note that most of the fouling was on the frame and the protecting mesh and less discernible on the polymer itself.

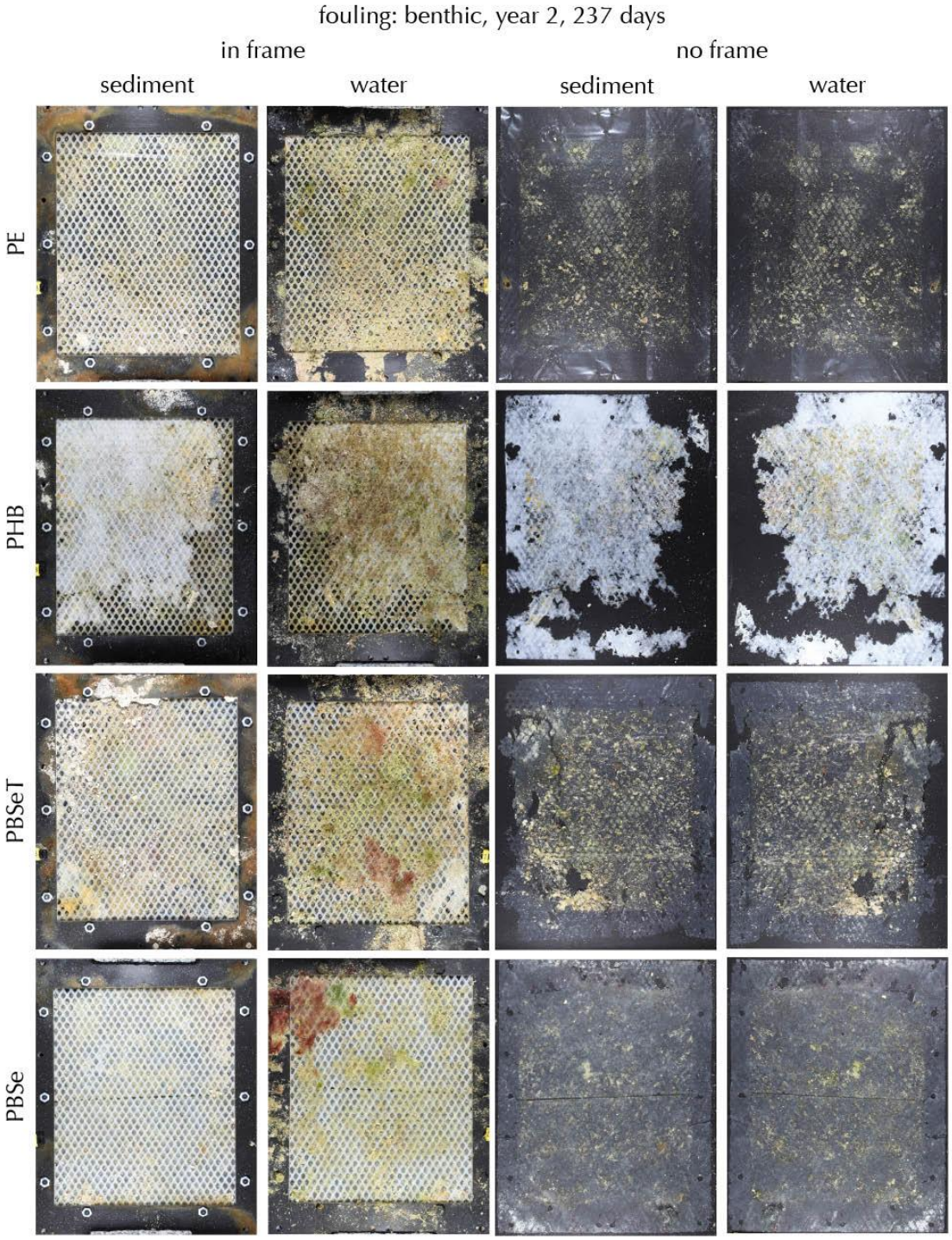


Figure 197. Exemplary images of LDPE, PHB, PBSeT and PBSe fresh samples from the mesocosm benthic (seafloor scenario) tests from year 2 after 238 days of exposure showed the biofilm formation (fouling). Left two columns are images of the polymer film still in the sample holder, right two columns show the polymers after their removal from the frames. From left, first and third columns show the side of the sample that was facing the sediment, second and fourth columns show the side that was facing the water and light. Note that most of the fouling was on the frame and the protecting mesh and less discernible on the polymer itself.

4.2.2 Proportional disintegration

The percentage of lost polymer was determined as area % from images of the dried samples, (Figure 198 up to Figure 203, and Appendix table 10). For comparison reasons the polymer film thickness was taken into account, and disintegration was also calculated to a volumetric rate as volume per area per day (10^{-6} cm^3 cm^{-2} d^{-1}). Samples from all polymers except LDPE in all three tested habitats eulittoral, benthic and pelagic were showing disintegration. The disintegration was gradually progressing with time, with two exceptions that are described separately below. About 90% disintegration was observed for PBSe and PBSeT in the eulittoral test in year 2 after 238 days (Figure 198 and Figure 201), and for PBSeT in the pelagic test in year 2 after 271 days (Figure 199 and Figure 202.). Most samples were disintegrated less than 50% after 308 respectively 270 days of exposure.

The rate of disintegration differed between habitats and polymers (Table 88 and

Table 89.), and was heterogeneous between replicate samples of the same habitat and polymer. This is expressed by a high standard deviation (error bars) in Figure 201 up to Figure 203 (see also Appendix table 10) and visualised exemplarily in Figure 201 and Figure 205. The mean rate of disintegration was highest in the eulittoral and lowest in the benthic tests for PBSeT in both years, and for PBSe in year 2. PHB disintegration was fastest in the benthic tests, and slowest in the year 1 experiment in the eulittoral tests, but in year 2 slowest in the pelagic tests.

Within the same habitat different polymers disintegrated at a different rate. PHB disintegrated faster than PBSeT in all three habitats in year 1. In year 2 in the eulittoral tests PBSe disintegrated fastest, and for PBSeT and PHB the disintegration rate was about the same. In year 2 in pelagic tests PBSeT disintegration was faster than for PHB, and PBSe disintegration was slowest. In year 2 in benthic tests the disintegration of PHB was faster than of PBSeT, and PBSe disintegration was slowest again.

Special cases

1) PHB pelagic tests

The disintegration of the PHB samples in the pelagic tests of year 1 and year 2 were heterogeneous within all samples in all time intervals that a general trend of a progressing degradation with time could not be observed (Figure 202.).

As the samples were unique and sacrificed at the sampling event the rate of disintegration can only be determined for each sample individually. Thus the timeline of disintegration of a certain polymer calculated as the mean from several samples is summing up variations of each individual experiment.

One reason for the heterogeneous disintegration of different individual samples might have been small-scale differences of environmental conditions within one mesocosm. In the pelagic test tanks the water was moved by pumps but the samples were at a fixed position within this water movement regime for the whole exposure time. Although the water was well mixed, spaces of higher and lower flow velocity are likely to have occurred as a result of a differentiation of flow at the obstacles, i.e. samples in their holders. Biofilms can change in growth and species composition along fine gradients and reflect the distribution and availability of transportable compounds of the medium. Also the lamps in the pelagic/benthic test tanks were not constructed in a way to create a perfectly even light regime. Small variations

due to reflection, shading and angle to the lamps might have caused heterogeneous illumination of the samples. Additionally, the samples were hanging in the water vertically so the upper part of the samples received more light than the lower part. Phototrophic organisms in the biofilm may have reacted to these differentiations of light and in an interplay with the differentiation of flow might have created a patchiness in biofilm composition. As a consequence, it is possible to assume a patchiness in biofilm activity which finally led to different disintegration rates of the individual samples.

2) PBSeT and PHB eulittoral tests in year 2

Samples of PBSeT and PHB in the eulittoral test in year 2 (Figure 191, blue columns) were disintegrated more after t_2 (152 d) and t_3 (238 d) than after t_4 (270 d). In the year 1 experiment disintegration was gradually progressing (Figure 191, grey columns) with exposure time. The samples were buried in sand for the whole exposure time and the sand was flushed twice a day with seawater, mimicking a tidal rhythm. For the experiment of the second year the tanks were filled with freshly-collected sediment. The experimental conditions between the two years' experiments was the grain size of the sediment used, and coupled to this the permeability. In year 1 the mean grain size was 278 µm, the mean permeability $17.9 \cdot 10^{-11}$ m^2. In year 2 the mean grain size was 206 µm, the mean permeability $9.54 \cdot 10^{-11}$ m^2. Linked to these sediment properties is also the retention capacity for the falling water during low tide where the water level is wandering horizontally though the sediment. It was noted during the sampling that the water was draining from the sediment at a lower rate in the year 2 experiment.

Although the reason is unknown, two technical observations could be helpful to interpret the lower disintegration in the t_4 samples. To avoid water being trapped within the sample holder on the polymer film the frames were buried in the sediment with a small angle of 11°. In some samples from both years there was a differentiation in disintegration within one specimen of polymer sample, along a gradient of water cover. This means the by 5 cm lower part of the sample disintegrated faster than the higher part (for example Figure 190 and Figure 191).

The second observation regards the geometry of the tanks used for the eulittoral test. The water circuit of the eulittoral tank was connected to the benthic/pelagic test tank below by a central tube perforated tube by which the porewater from the sediment could drain after the simulated high tide. Although being mechanically stable enough to hold approximately 275 kg of sediment and water the centre of the eulittoral tanks bent down for about 3 cm in the longitudinal axis. Deducing from these facts the hypothesis that regularly aerated polymer samples disintegrate slower than polymers that stay wet all the time we could explain the lower degradation rate in the series of samples of t_4 year 2 with the following scenario: Applying the same experimental conditions in terms of sediment depth, temperature, tidal rhythm the samples of the year 2 experiments at the margins of the tanks (t_1 and t_4) have been regularly exposed to air during low tide. The samples positioned centrally in the tank in year 2 may have experienced longer periods or permanent immersion in the porewater of the sediment. The reason may have been a higher rest water level in the centre part of the tanks at low tide thanks to the higher water retention capacity as a result of the higher capillary effect of the finer sediment (206 µm vs. 278 µm). Taking this into account the t_4 samples

(Figure 191) could have experienced the wanted experimental conditions (wet-dry rhythm) and thus showed about the same (expected/"normal") disintegration rates as in year 1 (Figure 190 and Figure 201). The t_2 and t_3 samples however could have experienced more humidity (or even water cover) than wanted and thus were faster in disintegration than t_4 samples. The effect of water cover/humidity on disintegration has to be further investigated systematically in order to assess the observed heterogeneous disintegration in the eulittoral tests. With these results a heterogeneity by design could be further minimised or avoided.

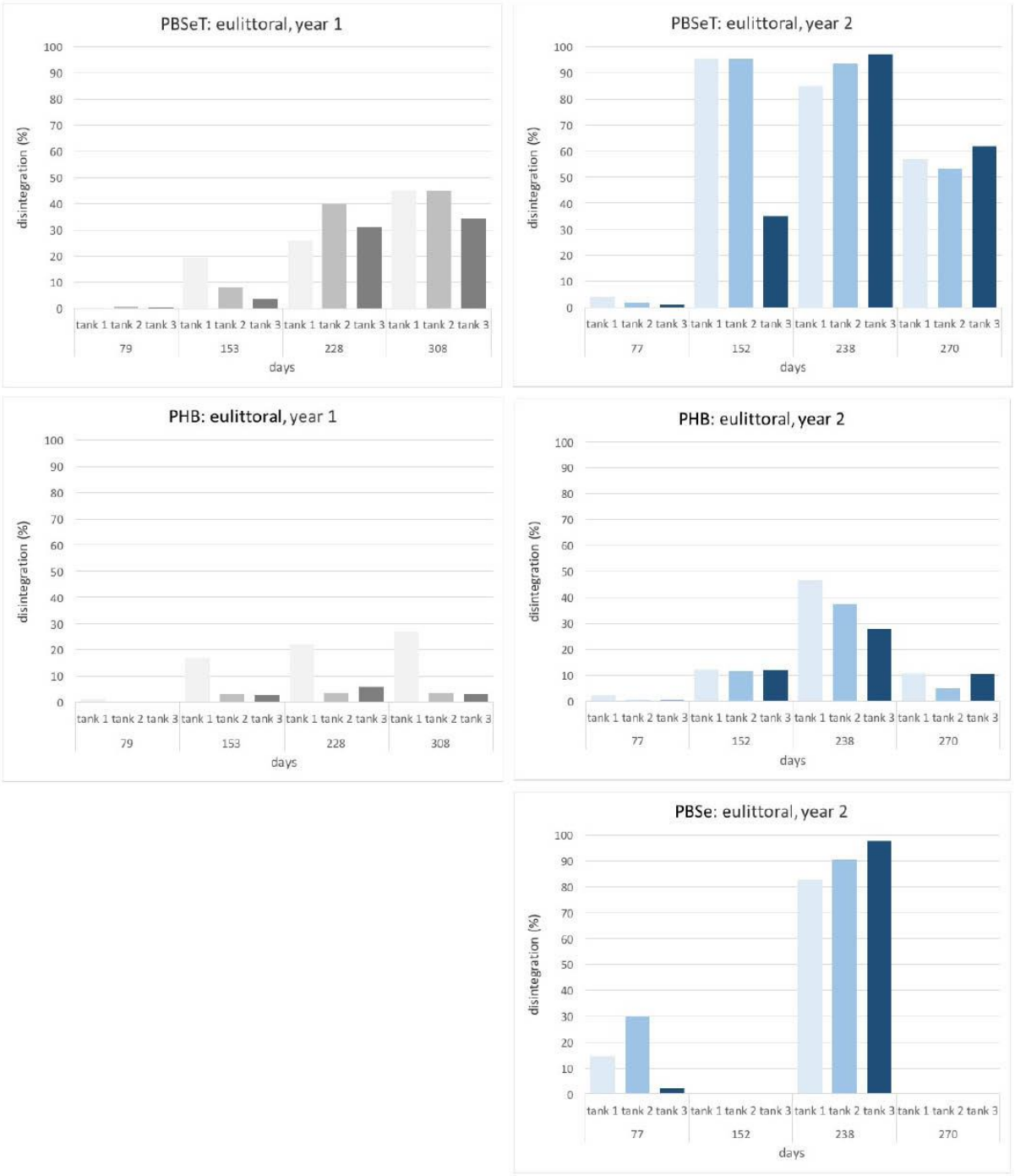


Figure 198. The disintegrated area of all PBSeT and PHB samples in all tanks from the mesocosm eulittoral tests (intertidal beach scenario) in the year 1 and the year 2 experiment. Data on PBSe samples from all tanks are available from year 2 at two sampling intervals (77 and 238 days). The exposure time is given on the x-axes.

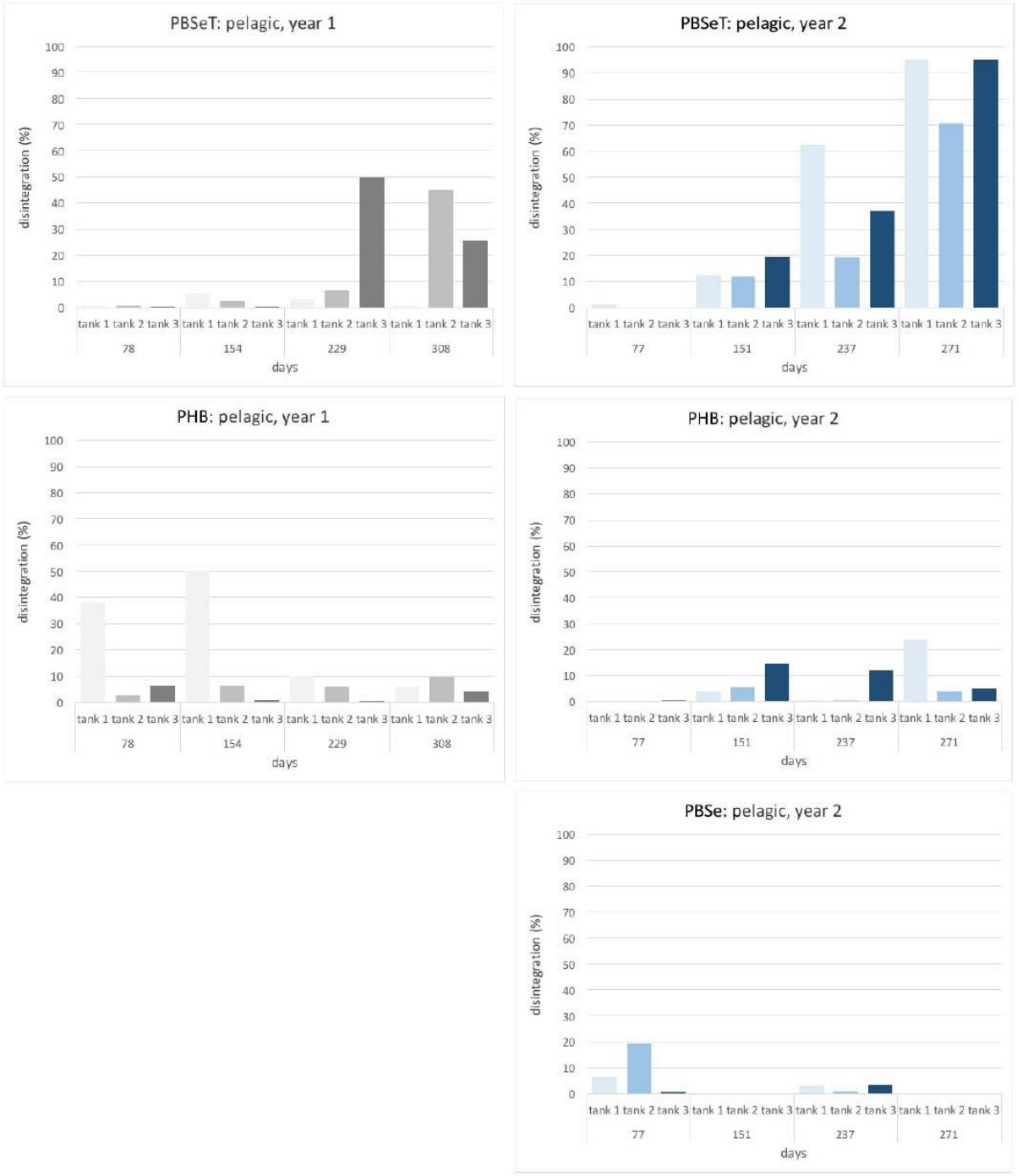


Figure 199. The disintegrated area of all PBSeT and PHB sample in all tanks from the mesocosm pelagic tests (water column scenario) in the year 1 and the year 2 experiment. Data on PBSe samples from all tanks are available from year 2 at two sampling intervals (77 and 237 days). The exposure time is given on the x-axes.

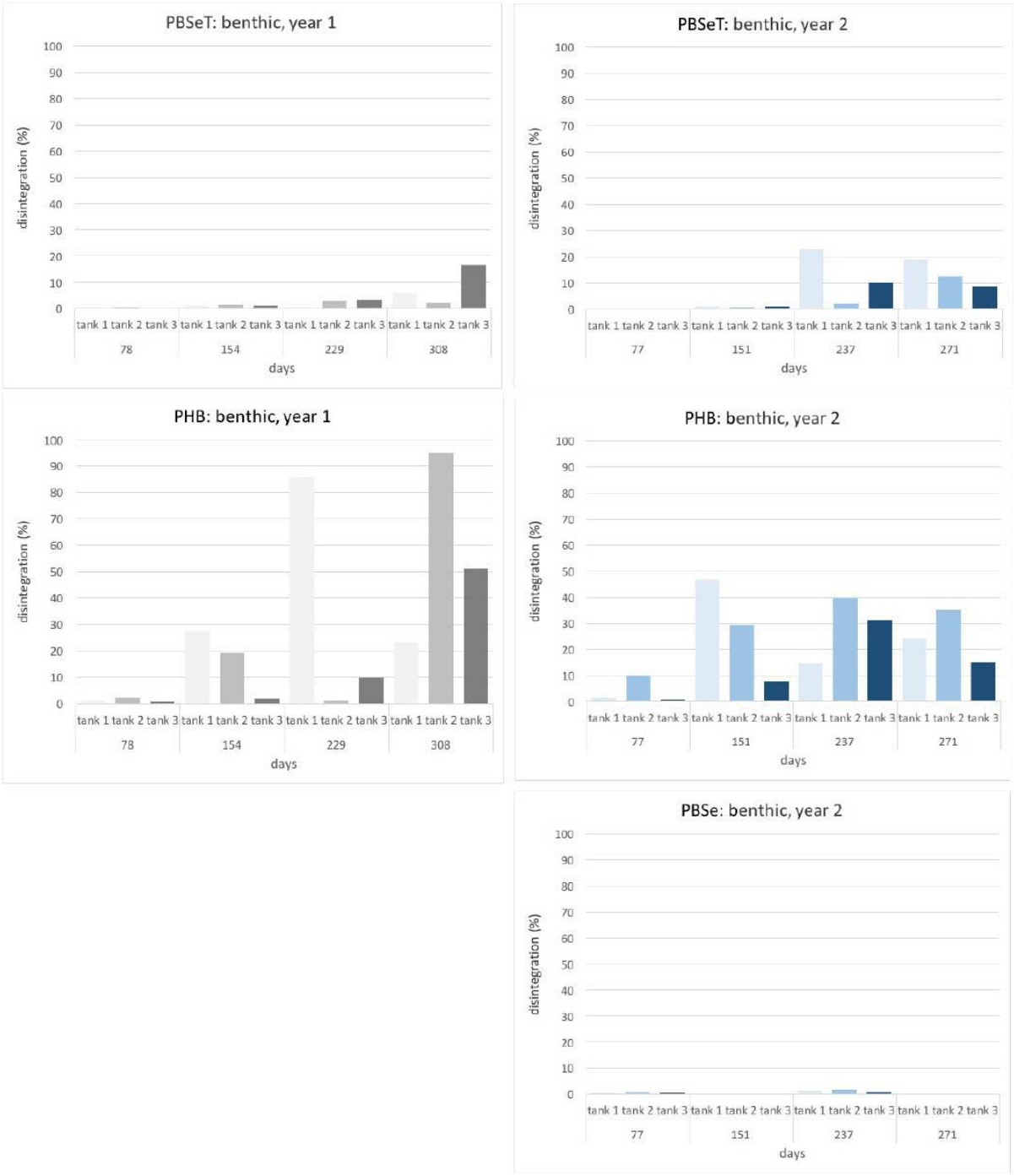


Figure 200. The disintegrated area of all PBSeT and PHB sample from all tanks from the mesocosm benthic tests (sublittoral, seafloor scenario) in the year 1 and the year 2 experiment. Data on PBSe samples from all tanks are available from year 2 and two sampling intervals (77 and 237 days). The exposure time is given on the x-axes.

Table 88. The disintegration rate of PBSe, PBSeT and PHB samples from the mesocosm eulittoral (intertidal beach scenario), pelagic (water column scenario) and benthic test (sublittoral, seafloor scenario) from year 1 and 2. The rate was corrected for the polymer film thickness of 25 µm for PBSe and PBSeT and 100 µm for PHB and is expressed in volume per area of film per day (10^{-6} cm^3 cm^{-2} d^{-1}) for comparability. Displayed is the mean value and its standard deviation of three replicates at 4 time points for PHB and PBSeT, and at 2 time points for PBSe. The negative control PE is not included.

	year 1			year 2		
	PBSe	PBSeT	PHB	PBSe	PBSeT	PHB
Eulittoral	-	2.18 ± 1.6	3.5 ± 3.9	9.927 ± 6.13	7.025 ± 5.2	7.0 ± 6.1
Pelagic	-	1.18 ± 1.73	9.4 ± 15.1	1.65 ± 2.38	3.7 ± 3.2	2.9 ± 3.4
Benthic	-	0.33 ± 0.35	11.1 ± 12.5	0.2± 0.08	0.65 ± 0.78	11.2 ± 8.5

Table 89. The ranking of the mean disintegration rate normalised for polymer film thickness (vol $area^{-1}d^{-1}$) of each polymer type between the habitats (left columns) and between the polymers in each habitat (right columns). The samples were exposed in the mesocosm eulittoral (intertidal beach scenario), pelagic (water column scenario), and benthic test (sublittoral, seafloor scenario) during year 1 and 2. The negative control PE is not included. n.a.: not available.

	between habitats			between polymers	
	year 1	year 2		year 1	year 2
PBSe	n.a.	E > P > B	Eulittoral	PHB > PBSeT	PBSe > PBSeT ≈ PHB
PBSeT	E > P > B	E > P > B	Pelagic	PHB > PBSeT	PBSeT > PHB > PBSe
PHB	B > P > E	B > E > P	Benthic	PHB > PBSeT	PHB > PBSeT > PBSe

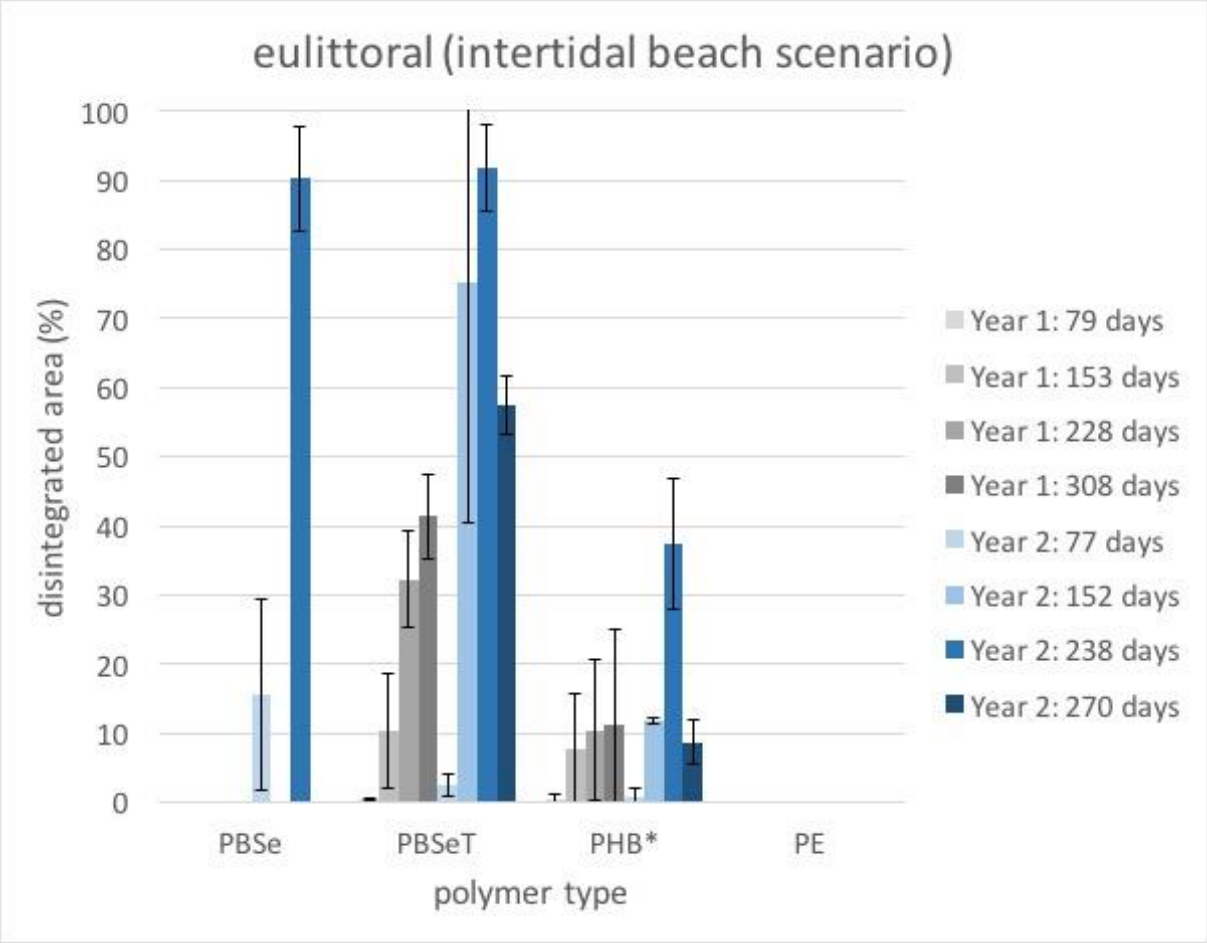


Figure 201. The disintegrated area (mean; n=3) of PBSe, PBSeT, PHB and PE samples from the mesocosm eulittoral test (intertidal beach scenario). The exposure time for each triplicate of samples is given in the legend. Error bars show the standard deviation. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

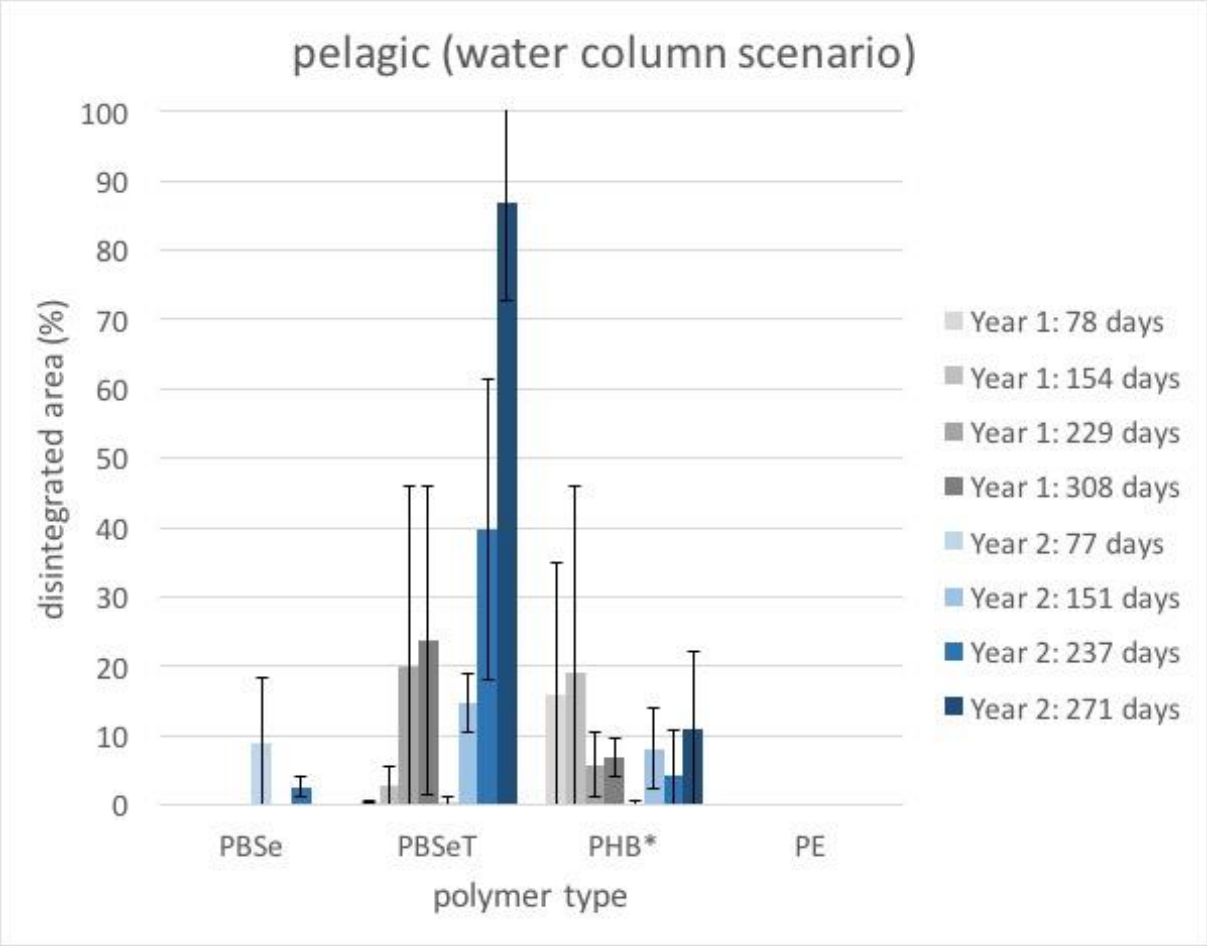


Figure 202. The disintegrated area (mean; n=3) of PBSe, PBSeT, PHB and PE samples from the mesocosm pelagic test (water column scenario). The exposure time for each triplicate of samples is given in the legend. Error bars show the standard deviation. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

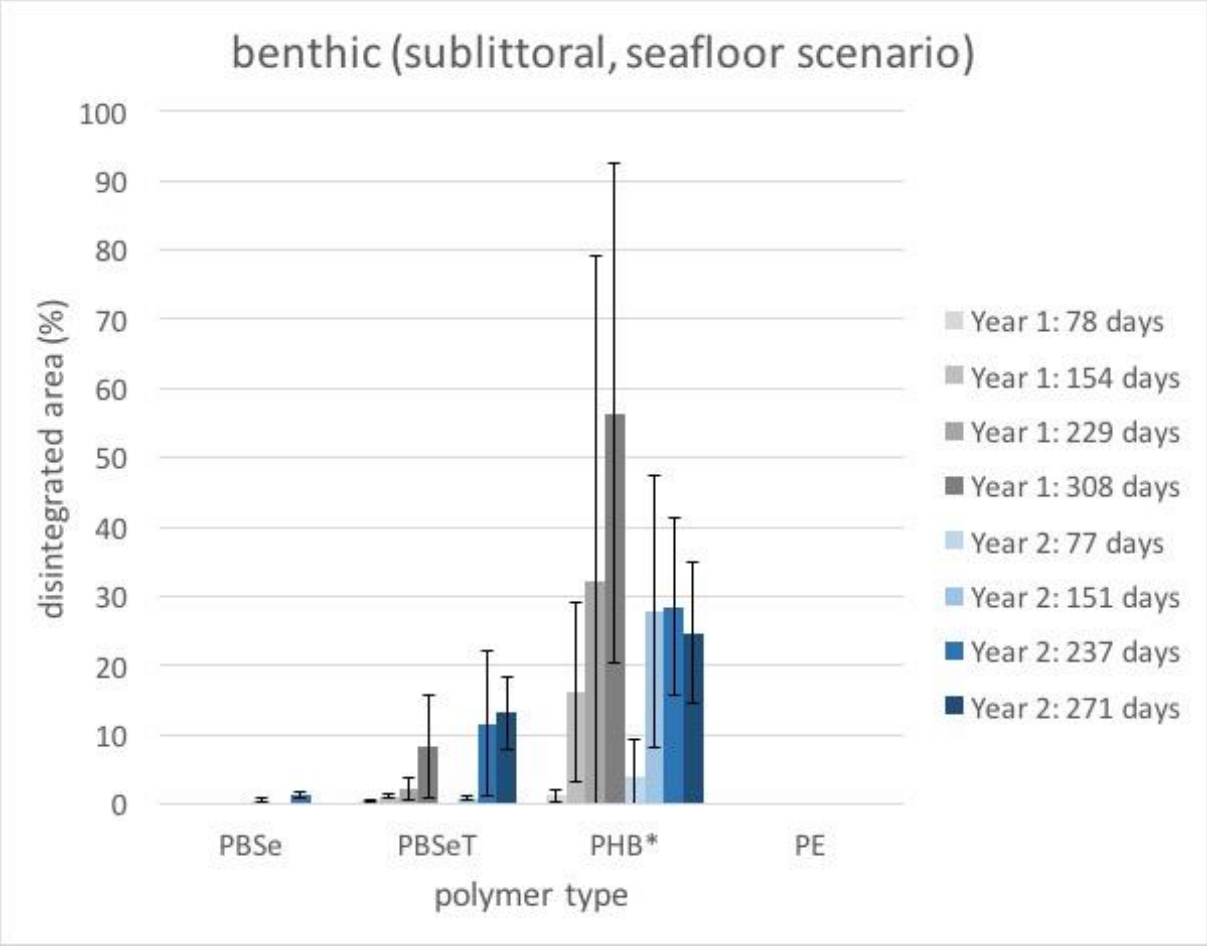


Figure 203. The disintegrated area (mean; n=3) of PBSe, PBSeT, PHB and PE samples from the mesocosm benthic test (sublittoral, seafloor scenario). The exposure time for each triplicate of samples is given in the legend. Error bars show the standard deviation. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

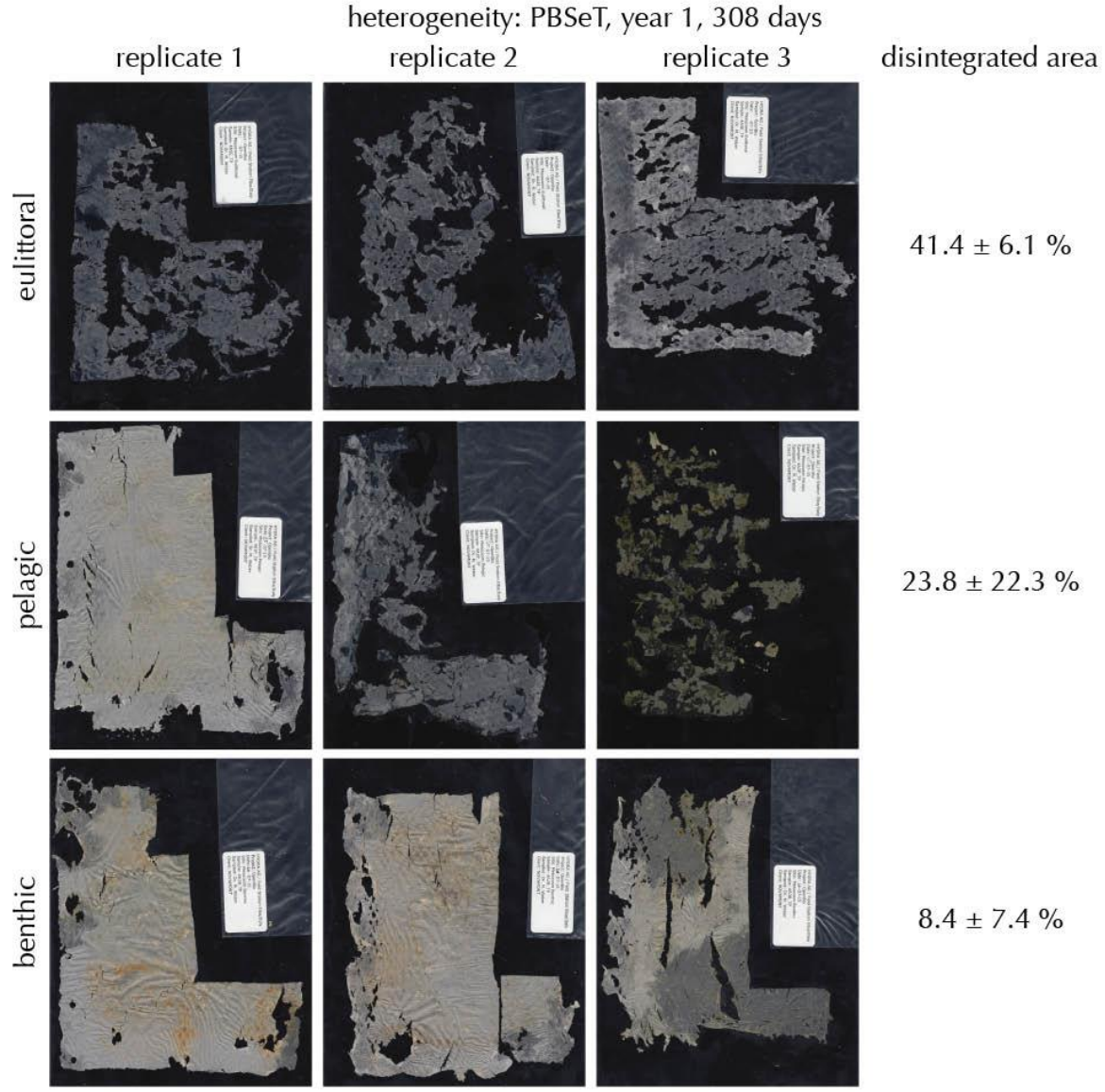


Figure 204. Exemplary scan images of PBSeT samples from the mesocosm eulittoral (intertidal, beach scenario), pelagic (water column scenario) and benthic test (sublittoral, seafloor scenario) from year 1 showed heterogeneous disintegration between the three replicates. The exposure time was 308 days. Variability was low within the replicates of the samples from the eulittoral (41.4 ± 6.1 %) and high within the samples from the benthic (8.4 ± 7.4 %) and the pelagic tests (23.8 ± 22.3 %).

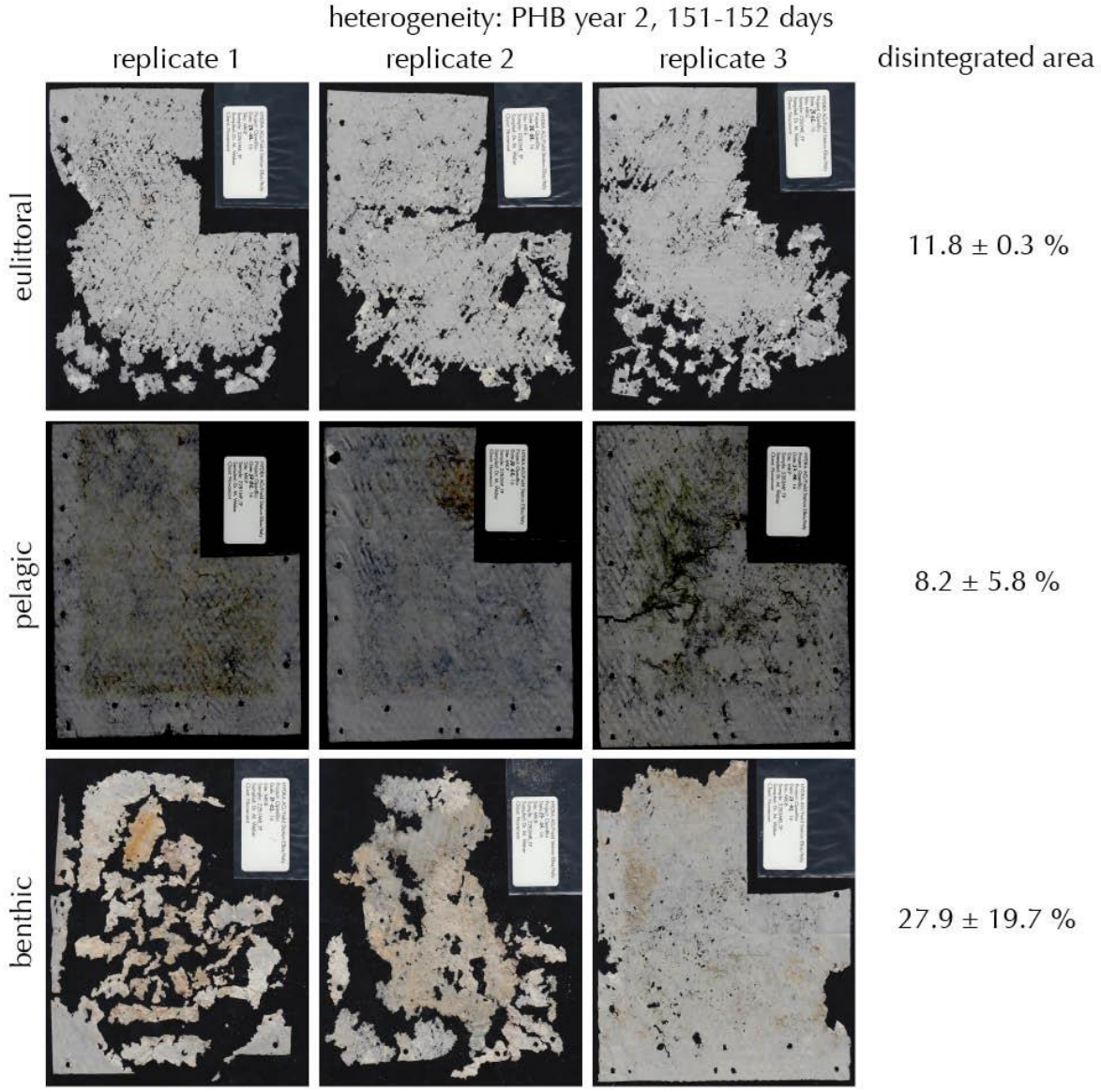


Figure 205. Exemplary scan images of PHB samples from the mesocosm eulittoral (intertidal, beach scenario), pelagic (water column scenario) and benthic test (sublittoral, seafloor scenario) from year 2 showed heterogeneous disintegration between the three replicates. The exposure time was 151-152 days. Variability was low between the replicates of the samples from the eulittoral (11.8 ± 0.3 %) and high within the samples from the benthic (27.9 ± 19.7 %) and the pelagic tests (8.2 ± 5.8 %).

4.2.3 Tensile property, GPC, and MALDI-TOF data

MALDI-TOF

For PBSe the mass spectra of the untreated samples and all test samples showed ion distributions with the repetitive unit of 256.1669 Da corresponding to polybutylene sebacate ($C_{14}H_{24}O_4$) (Appendix figure 1 to Appendix figure 3). For PBSeT the mass spectra of the untreated samples and all test samples showed ion distributions with two repetitive units (Appendix figure 4 to Appendix figure 6). One of 256.1669 Da corresponding to polybutylene sebacate ($C_{14}H_{24}O_4$), and one of 220.0730 Da corresponding to butylene terephthalate ($C_{14}H_{12}O_4$). There were several polymeric distributions observed for PBSe and PBSeT, but the difference between all the ion distributions found is the mass of the end chains (Appendix table 11). The ion distributions in the spectra of all mesocosm samples analysed was similar to the untreated polymer samples (Appendix table 12 and Appendix table 1). There was also no difference in the mass of the repetitive units observed, meaning no cut had occurred in the repetitive units of PBSe and PBSeT.

Based on the MALDI-TOF results no chemical degradation of the polymers could be determined.

GPC analysis

Figure 206 shows the chromatograms of the PBSe native material (time 0) and after 9 months of exposure, in order to follow its molecular decay. The curves for all the environments overlapped, indicating that there weren't differences in molecular weights between the different samples. Also for PBSeT no relevant modifications of the molecular weight were recorded after 7.5 months of exposure (Figure 207). The slight differences between the curves (initial polymer vs. degraded polymers) were considered not relevant.

The direct consequence of these results is that this analytical approach was considered not suitable to follow and described the polymeric degradation and no further analyses were performed.

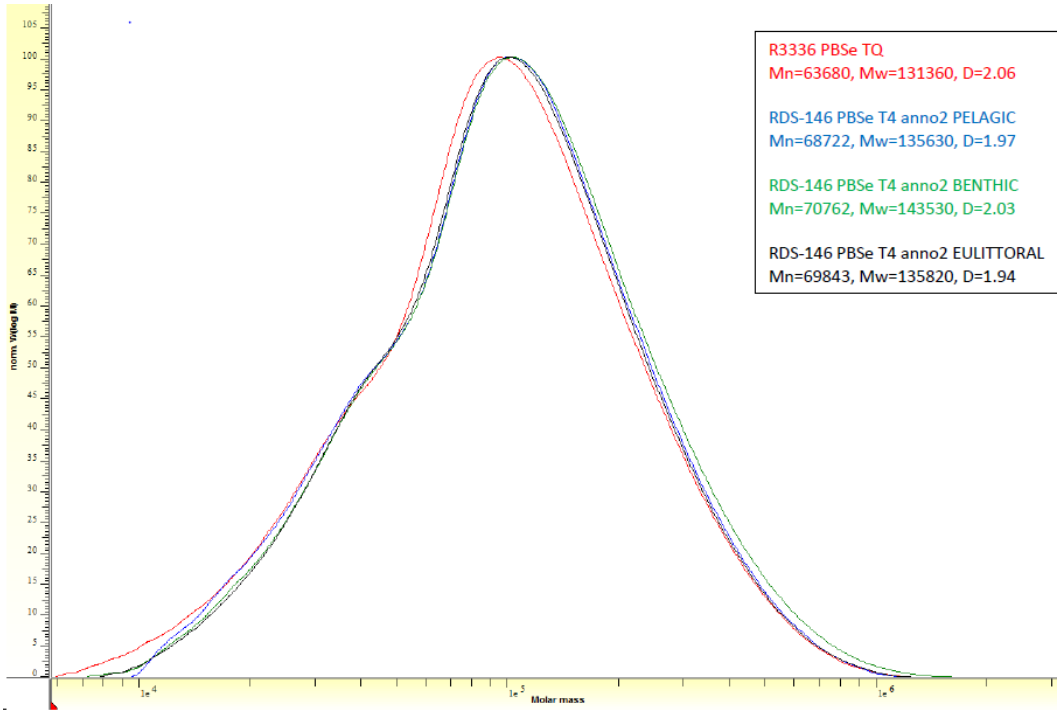


Figure 206. The molecular weight, analysed by GPC technique, of a PBSe native sample (red line) and samples exposed for 9 months in all the three habitats: blue line=pelagic (water column scenario), green line=benthic (seafloor scenario), and black line=eulittoral (intertidal scenario).

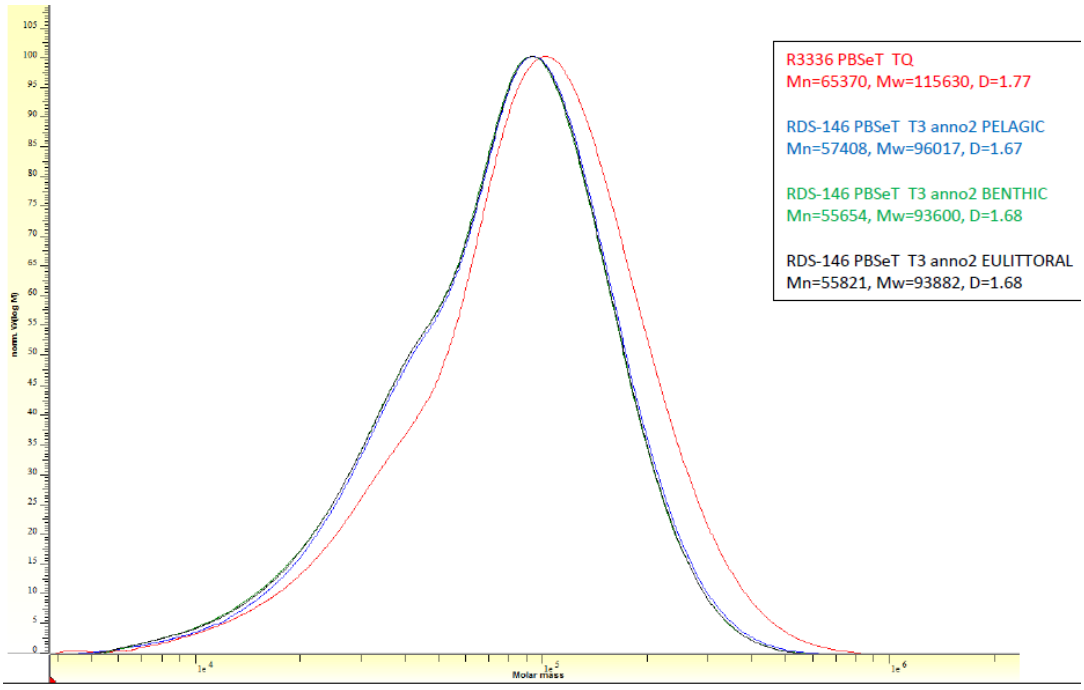
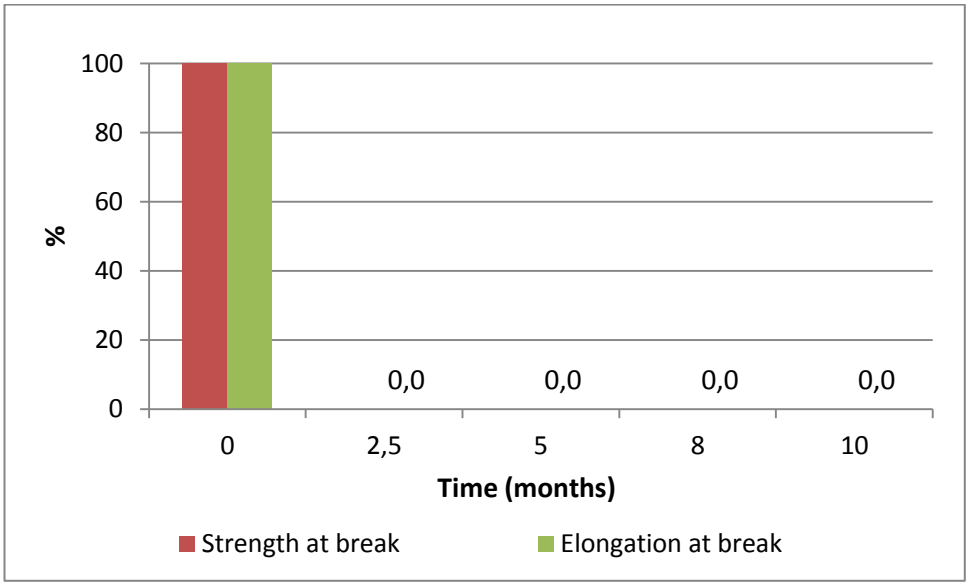


Figure 207. The molecular weight, analysed by GPC technique, of a PBSeT native sample (red line) and samples exposed for 7.5 months in all the three habitats: blue line=pelagic (water column scenario), green line=benthic (seafloor scenario), and black line=eulittoral (intertidal scenario).

Tensile properties measurements (year 1)

In Figure 208 were reported the tensile properties, of the eulittoral environment, measured as percentage decrease where the initial value was referred to 100%. The tensile properties revealed a high activity of mesocosm concerning the eulittoral environment. PBSeT, PHB and LDPE tensile properties were represented. PBSeT test material highlighted a total decrease of its tensile properties from the first sampling time. Is important to note that the test material did not disintegrated totally from the first sampling but only was characterized by the presence of countless cuts that make it impossible the measuring of tensile properties. At the same way PHB test material showed a decrease of its tensile properties linked to the progressive sampling till became zero from the month 8. In this case the elongation at the beak decreases more quickly than the strength at break. Is important also remember that the PHB test material is characterized by a higher thickness if compared with PBSeT (100 vs. 25 µm). Concerning the LDPE test material (negative control) is possible to note that the tensile properties remaining constant during all the length of the test. Only a slight decrease of elongation at the break parameter is recorded but no decrease trend is detectable. In Figure 209 were reported the tensile properties, of the benthic environment, measured as percentage decrease where the initial value is referred to 100%.

PBSeT



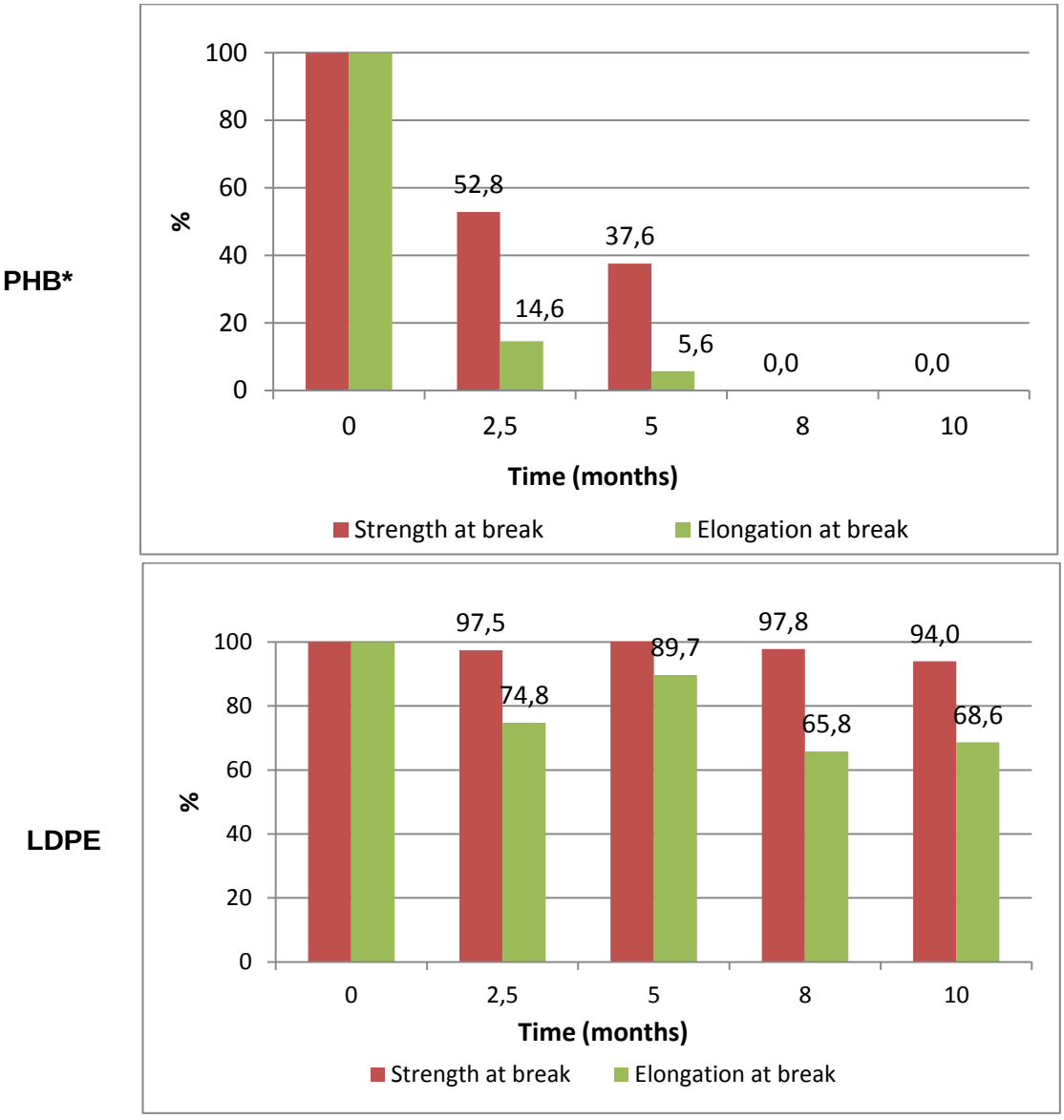
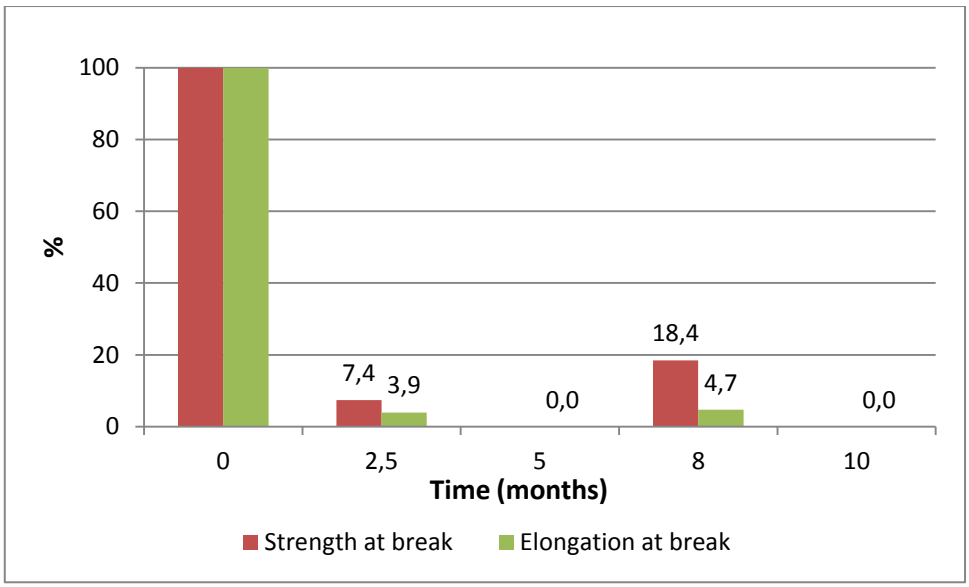


Figure 208. The tensile properties of PBSeT, PHB* and LDPE samples from the mesocosm eu-littoral test (intertidal beach scenario) of year 1. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSeT was 25 µm.

Shown in Figure 209 the tensile properties revealed a lower activity of mesocosm concerning the benthic environment if compared to the eulittoral ones. PBSeT, PHB and LDPE tensile properties were represented. PBSeT test material highlighted a strong decrease (near to the totality) of its tensile properties from the first sampling time. Is important to note that the test material did not disintegrated totally from the first sampling but only was characterized by the presence of countless cuts that make it impossible the measuring of tensile properties. This behaviour was identical to the eulittoral environment. At the same way PHB test material showed a pronounced decrease of its tensile properties linked to the progressive sampling. The sample coming from the month 5 was not possible to measure due to the high disintegration that was not present in the sample characterized by a higher time (month 8 and 10). In this case the elongation at the beak decreases more quickly than the strength at break but the values remaining stable along the whole test. Also in this environment, PBSeT test material, highlighted a quicker disintegration speed if compared to the PHB test material (probably due to the lower thickness). Regarding the LDPE test material (negative control), is possible to note that the tensile properties remains constant during all the length of the test. Only a slight decrease of elongation at the break parameter was recorded but no decrease trend was detectable.

PBSeT



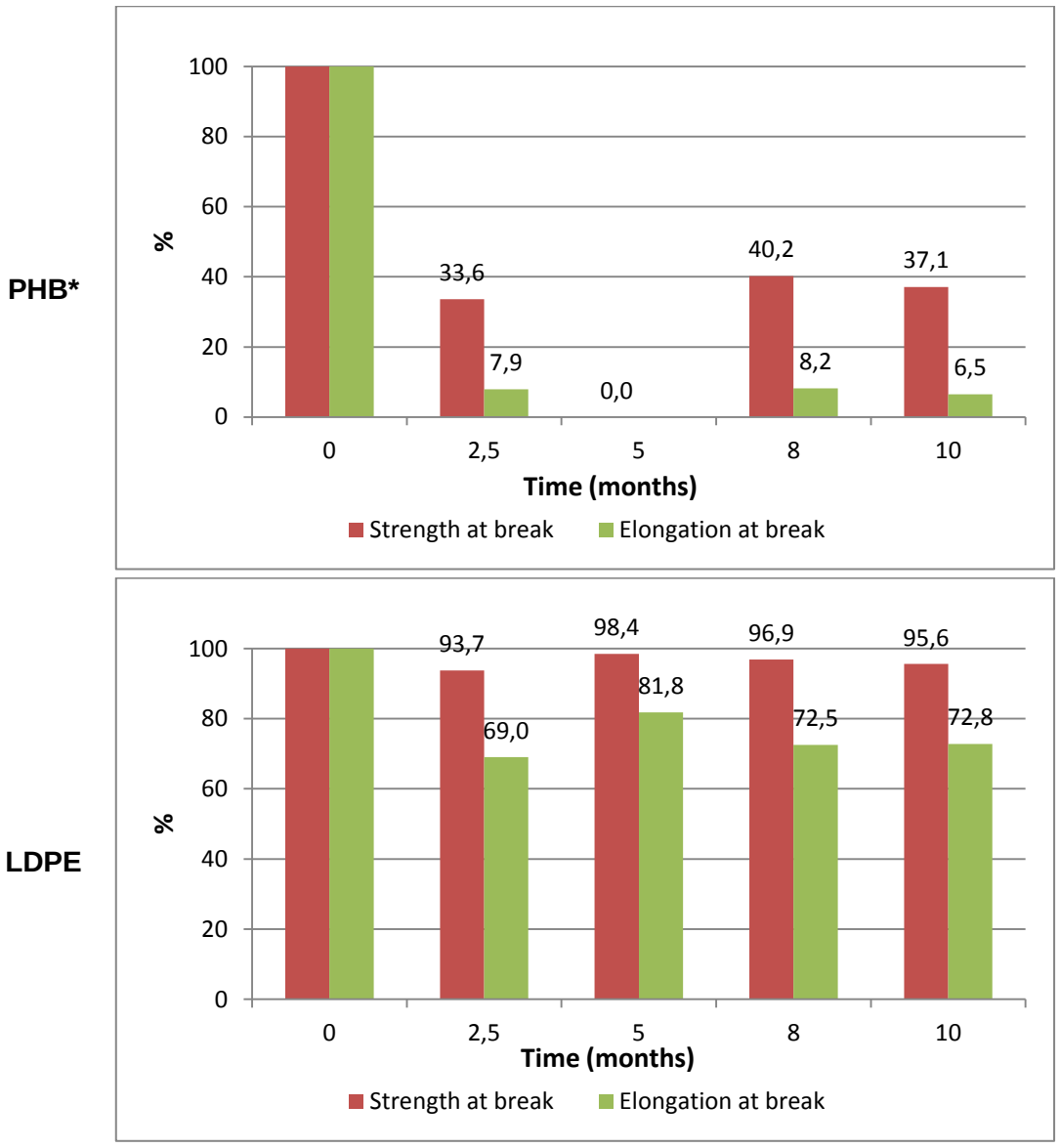


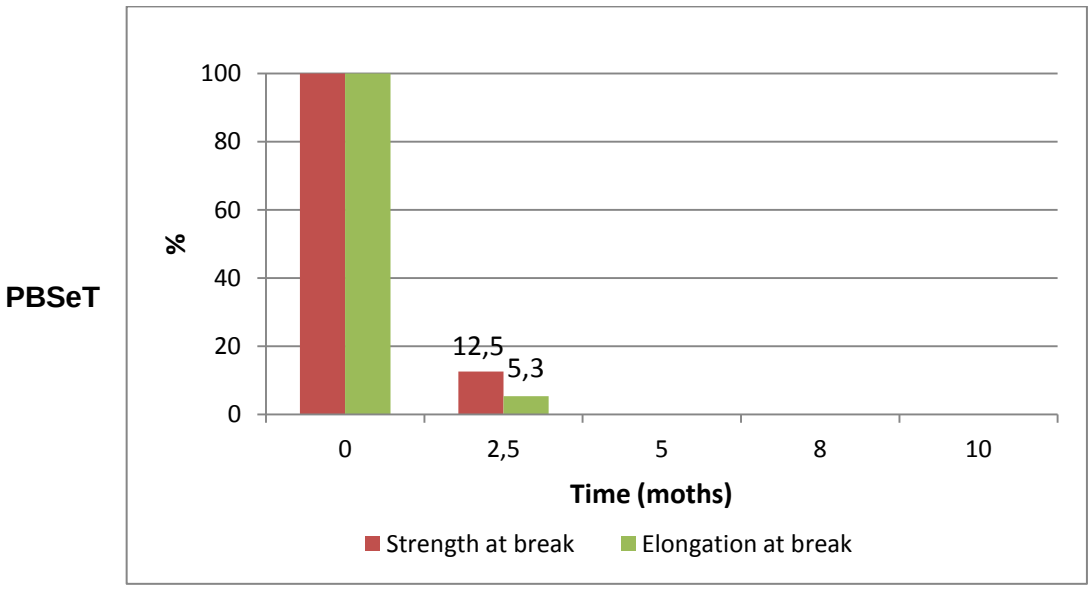
Figure 209. The tensile properties of PBSeT, PHB* and LDPE samples from the mesocosm benthic test (sublittoral seafloor scenario) of year 1. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSeT was 25 µm.

Shown in Figure 210 the tensile properties revealed a highest activity of mesocosm concerning the pelagic environment if compared to the eulittoral and benthic ones. PBSeT, PHB and LDPE tensile properties were represented. PBSeT test material highlighted a strong decrease (near to the totality) of its tensile properties from the first sampling time till to reach the zero point at the month 5. Is important to note that the test material did not disintegrated totally from the first sampling but only was characterized by the presence of countless cuts that make it impossible the measuring of tensile properties. This behaviour was identical as happened to the eulittoral and benthic environment. At the same way PHB test material showed a total decrease of its tensile properties from the first sampling time. Pelagic represent the more active environment for PHB test material despite its higher thickness. Concerning the LDPE test material (negative control) is possible to note that the tensile properties remaining constant during all the length of the test. Only a slight decrease of elongation at the break parameter was recorded but no decrease trend was detectable.

In general, the mechanical properties determination of the sample coming from the year 1 test, showed as the mesocosm was active on the degradation of the test specimens. After 2,5 months a total loss of mechanical properties is observed in Mesocosm-eulittoral and in Mesocosm-pelagic habitat. The decay of PHB is in general more gradual this is due to the high thickness of the sample. How expected LDPE samples had a negligible or limited elongation at break that showed a decrease of about 30% in all conditions. It is possible to summarize the decay of the mechanical properties:

PBSeT: Eulittoral > Pelagic and Benthic

PHB: Pelagic > Eulittoral > Benthic



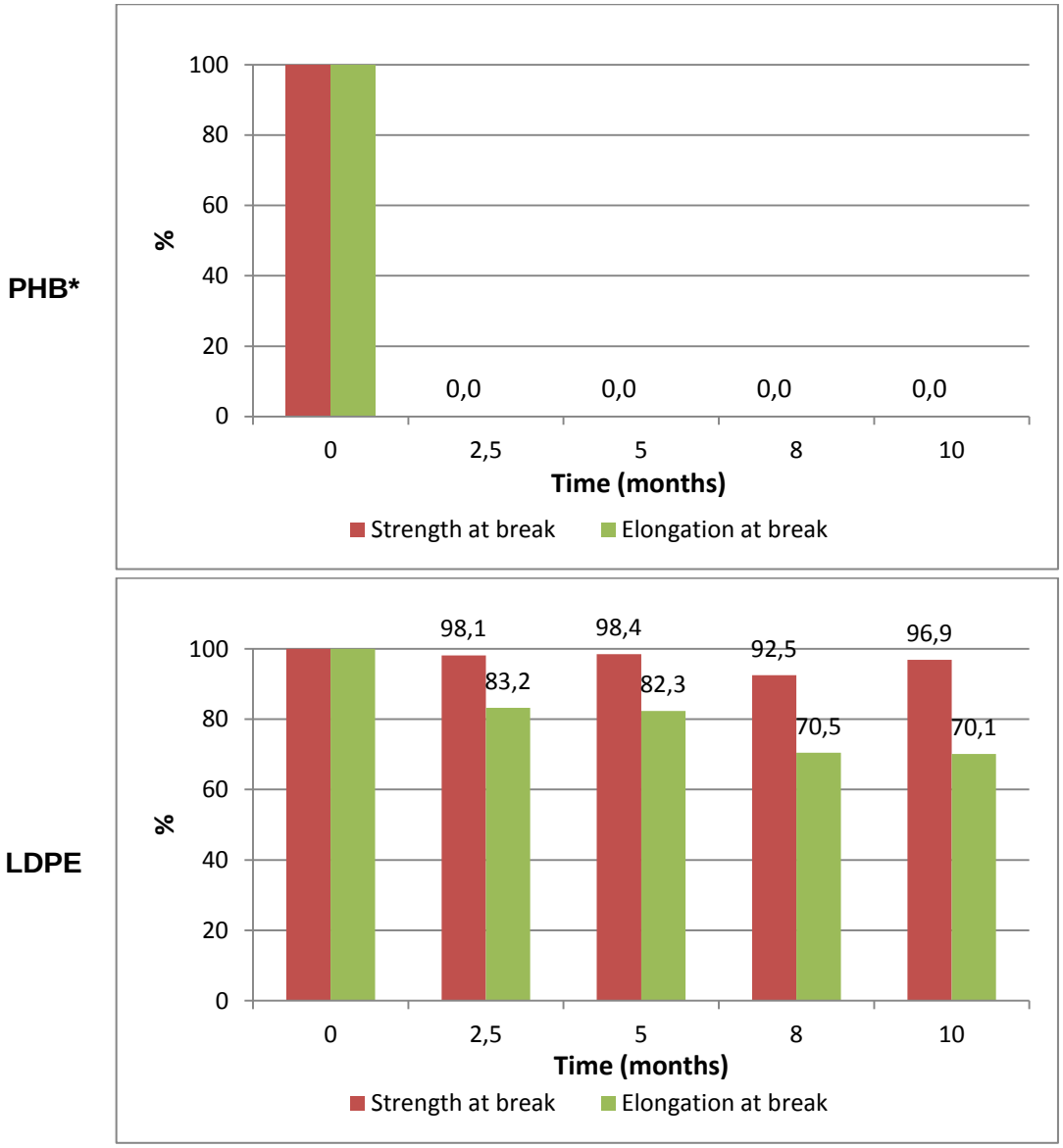
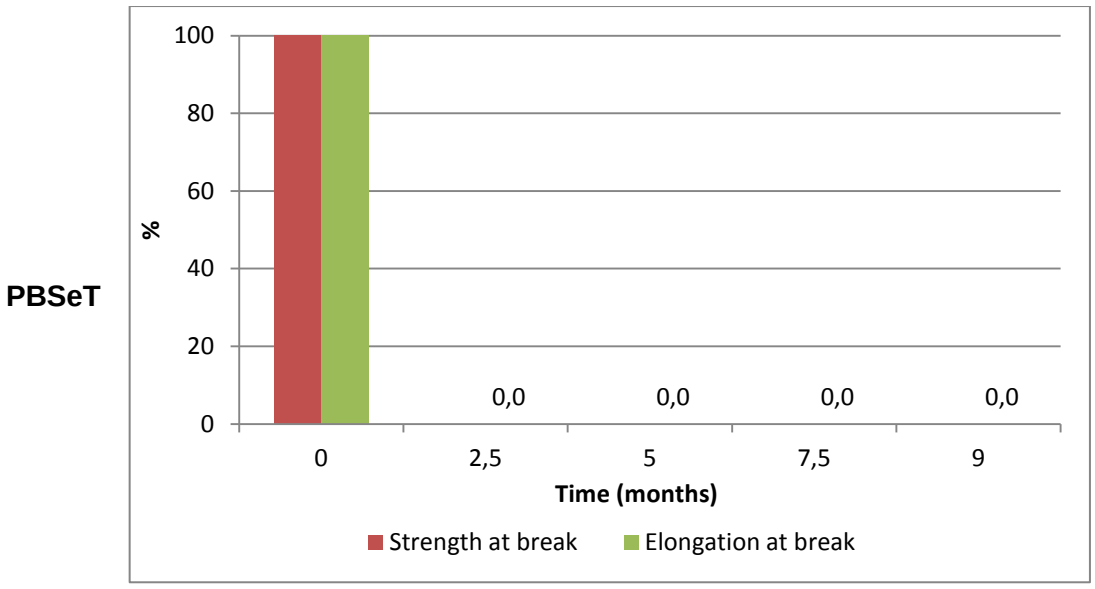


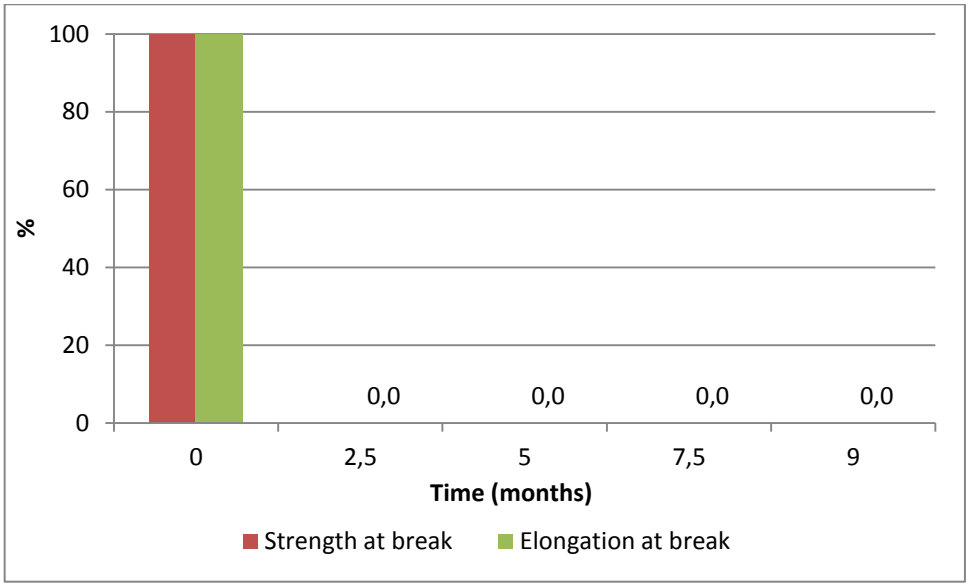
Figure 210. The tensile properties of PBSeT, PHB* and LDPE samples from the mesocosm pelagic test (water column scenario) of year 1. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSeT was 25 µm.

Tensile properties measurements (year 2)

To confirm the results obtained during the first year of mesocosm experimentation, has been performed a second cycle of disintegration of polymers (year 2) and the mechanical properties were measured as performed during the first year. Results obtained from the analysis of the test materials are reported in this section. As happened during the first year also in the second year of test, the tensile properties decreased very quickly highlighting their limit to follow the disintegration process. In the eulittoral mesocosm environment (Figure 211) the thinner materials (PBSeT and PBSe) showed a total loss of the mechanical properties from the first sampling point (2,5 months). It was possible measure only the PHB (time 1), probably due to its higher thickness. Notwithstanding that, the strength at break was only the 46% and the elongation at break the 9% if compared to the initial polymer value (time 0). From (and including) the second sampling time was not possible measure these parameters due to the excessive polymer fragmentation. It is important to note that the test material did not disintegrated totally from the first sampling but only was characterized by the presence of countless cuts that make it impossible the measuring of tensile properties. LDPE test material was always recovered and the tensile properties were measured. The graph shows that the strength at the break did not change and that the elongation at the break suffered of a preliminary reduction (about the 35%) that remain constant during the whole test duration.



PBSe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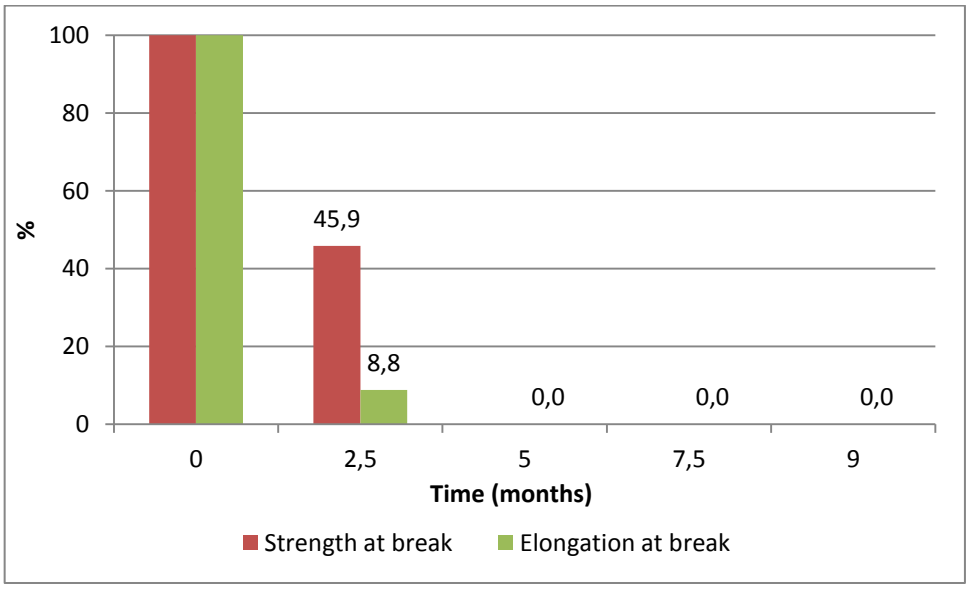
Time (months)
Strength at break
Elongation at break

PHB*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Time (months)
Strength at break
Elongation at break

LDPE

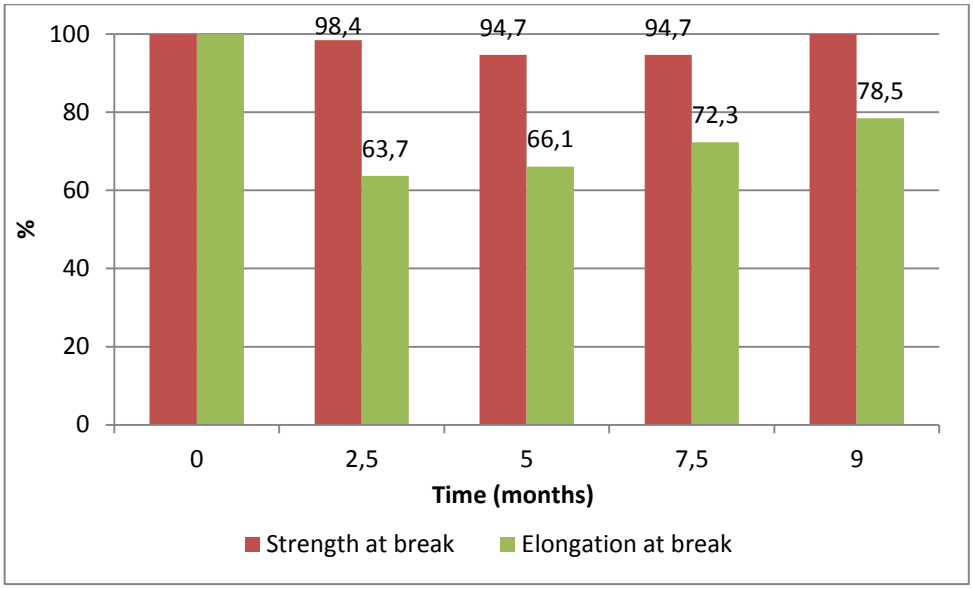
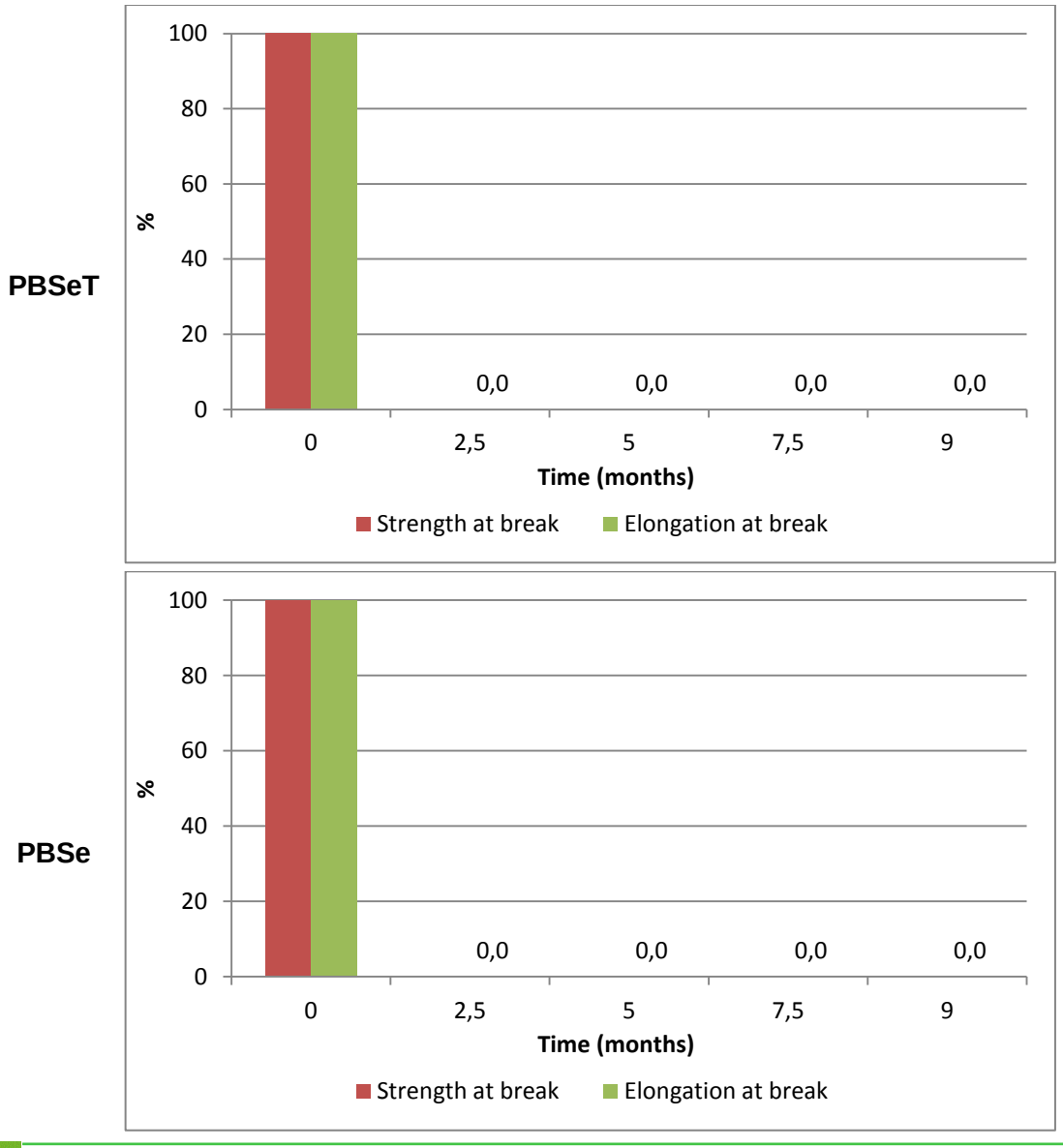


Figure 211. The tensile properties of PBSe, PBSeT, PHB* and LDPE samples from the mesocosm eulittoral test (intertidal beach scenario) of year 2. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

The benthic environment (Figure 212) showed the same activity, if compared to eulittoral environment, concerning the fast decrease of the tensile properties. As reported, the PBSe and PBSeT test material lost their mechanical characteristics from the first sampling time. PHB test material remained measurable only for the first sampling point but results highlighted a very strong reduction of strength and elongation at the break (-80% and -97% respectively). From the second sampling time was not possible measure the tensile properties due to the high fragmentation of the specimen. Is important to note that the test materials did not disintegrated totally from the first sampling but only were characterized by the presence of countless cuts that make it impossible the measuring of tensile properties. LDPE highlighted also in this environment its stability and the tensile properties showed as the reduction of strength and elongation at the break were stable from the first sampling to the end of the test. The decrease was comparable to that one measured in the eulittoral environment.



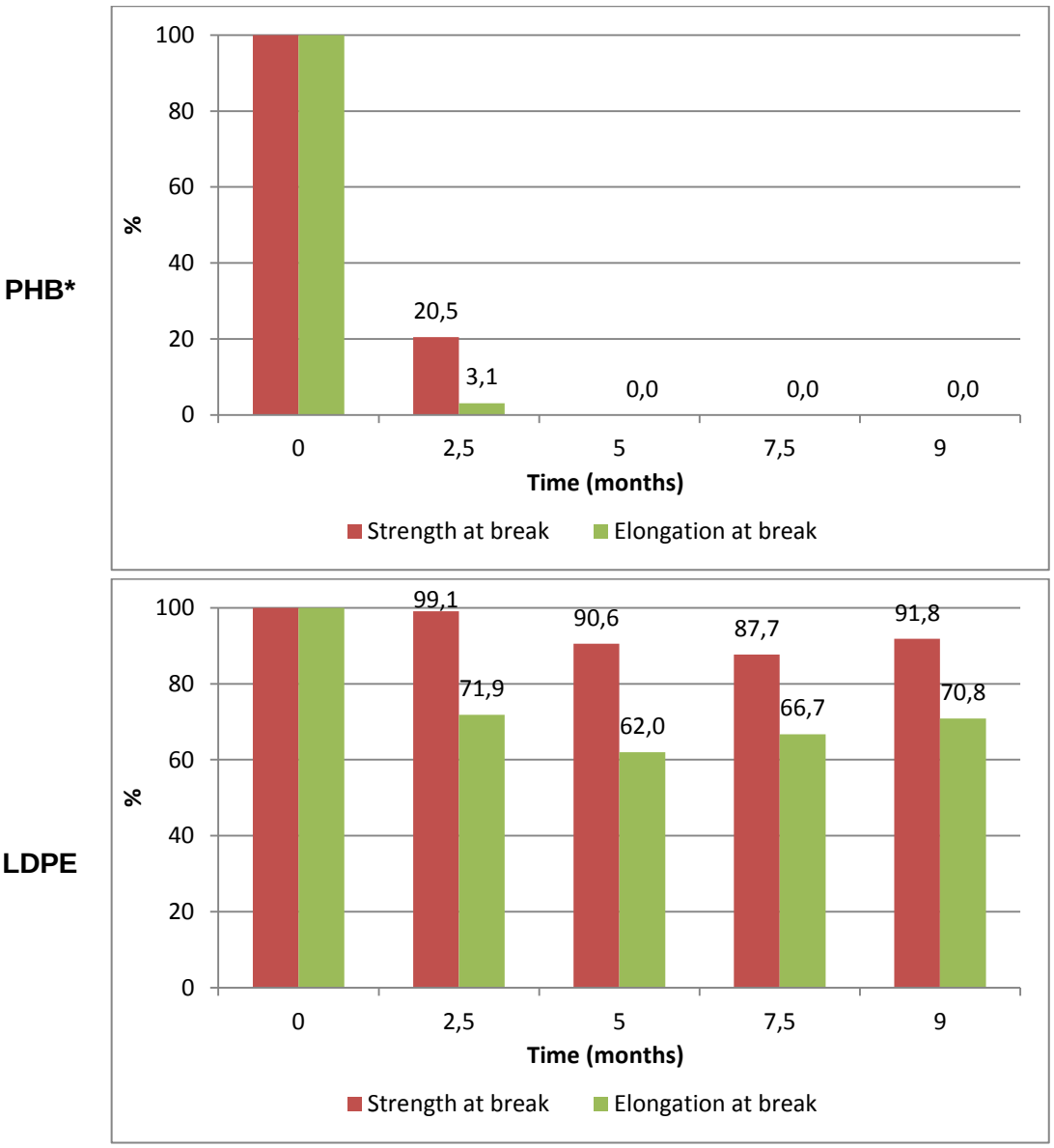


Figure 212. T The tensile properties of PBSe, PBSeT, PHB* and LDPE samples from the mesocosm benthic test (sublittoral seafloor scenario) of year 2. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

The tensile properties measurement concerning the pelagic environment were reported in Figure 213. Comparing these results with the eulittoral and the benthic environments, it is possible to note that the pelagic zone is less active (in this second year) and the test materials were measurable for more sampling times. In detail PBSeT highlighted a strong decrease (near to 100% for both parameters) from the first sampling point. PBSe test material showed a total decrease of the elongation at the break parameter but it maintained a stable reduction (more than 80%) of strength at break during the first and second sampling point (until 5 months of exposure). From the third sampling point any tensile properties have been possible to measure. PHB showed the same general behaviour of PBSe where the higher decrease of tensile properties was measured at the first sampling and after that the values remaining constant during the second and also for the third sampling point. In detail was registered a decrease around the 60% of the strength at the break and of the 92-95% for the elongation at the break parameter. LDPE test material, as expected, showed the same behaviour that the previous environments (both) where a restrained decrease of the tensile properties was registered and maintained for all the test length.

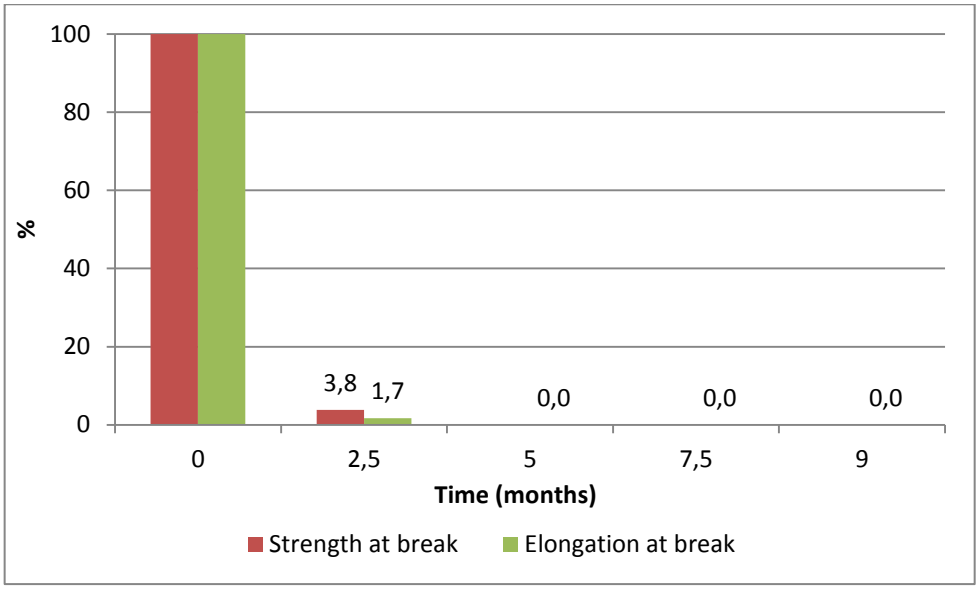
In general, also during the second year, the mechanical properties showed that the mesocosm was active and able in disintegrate the polymer samples. The polyesters (PBSe and PBSeT) highlighted a fast decay in the eulittoral and benthic environment. The pelagic environment tends to be less aggressive but very limited differences were recorded. The decay of PHB is in general more gradual probably due to the high thickness of the specimen. How expected on LDPE sample, the mechanical degradation is negligible or limited to the elongation at break that showed a decrease of about 30% in all conditions. The results of the second year are comparable to how observed during the first year test. The only exception was registered with the PHB in the pelagic environment, where the disintegration was more slow than in the first year.

It is possible to summarize the mesocosm activity on the decay of the mechanical properties of the second year as follow:

PBSeT and PBSe: Eulittoral and Benthic are more active than Pelagic

PHB: Benthic > Eulittoral > pelagic

PBSeT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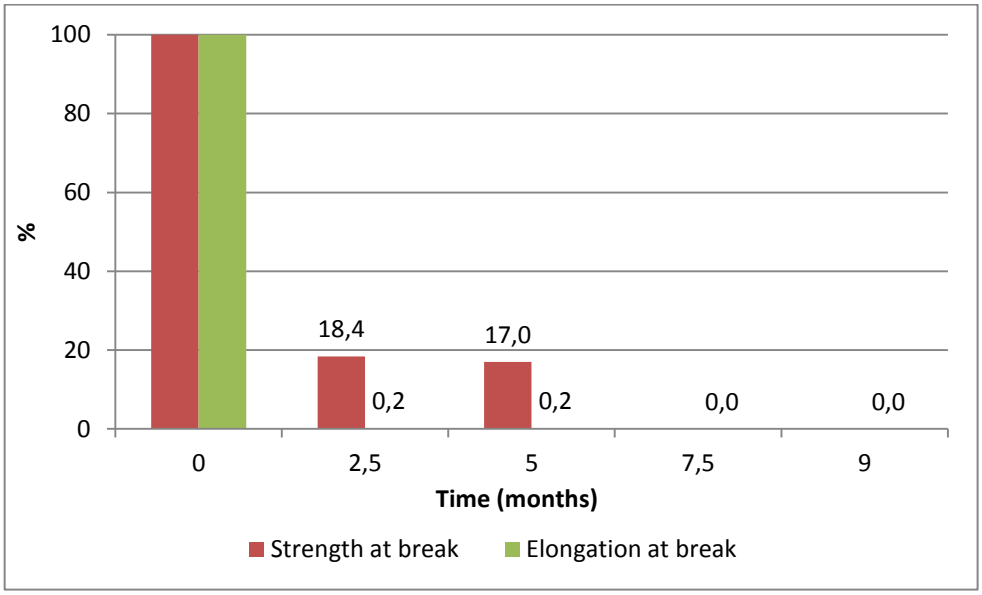
9
Time (months)
Strength at break
Elongation at break

PBSe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0
%
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Time (months)
Strength at break
Elongation at break

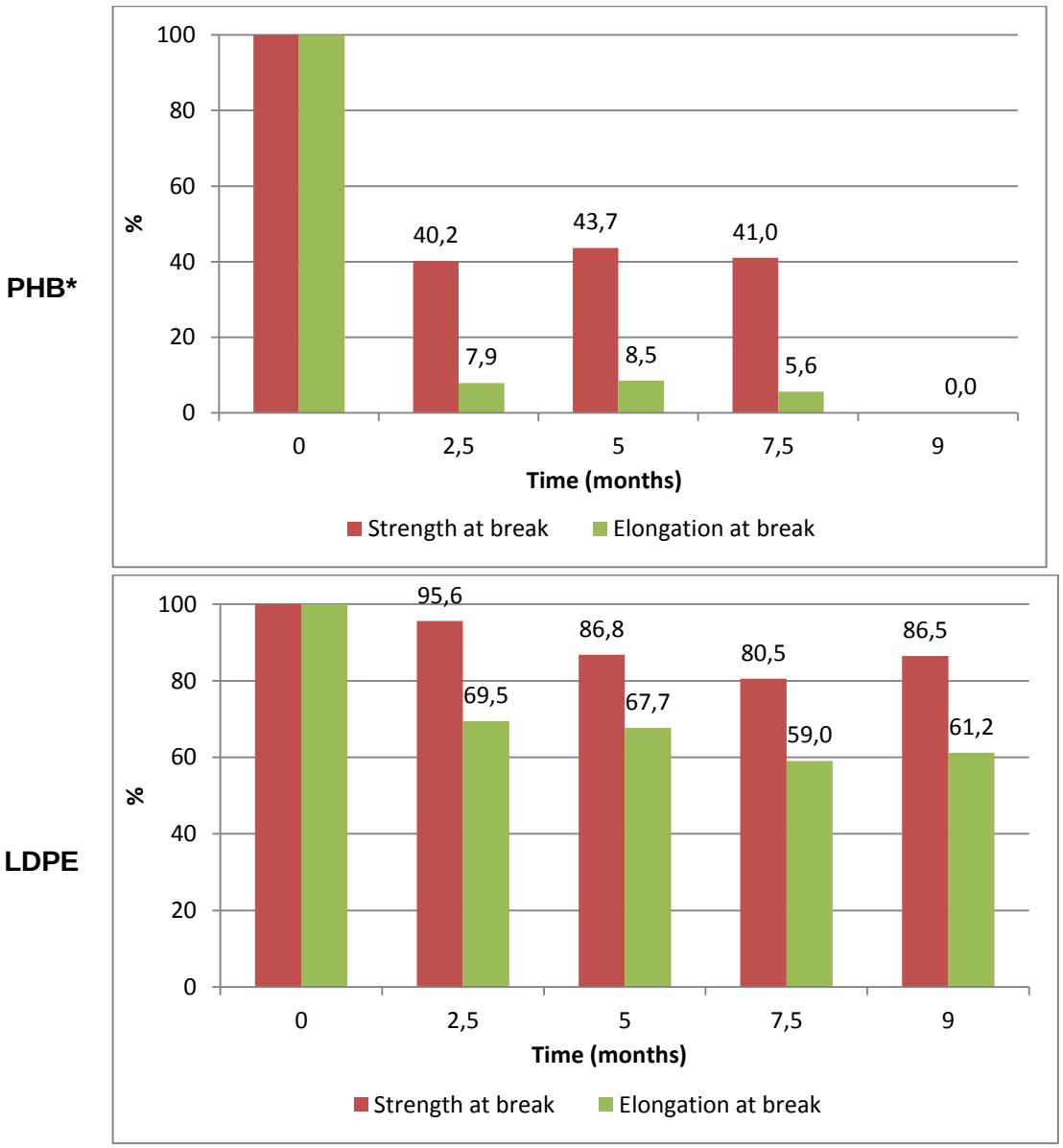

Figure 213. The tensile properties of PBSe, PBSeT, PHB* and LDPE samples from the mesocosm pelagic test (water column scenario) of year 2. For single data and the standard deviation see Appendix table 14. *Note: PHB film thickness was 100 µm, that of PBSe and PBSeT was 25 µm.

5 Discussion

5.1 Is the proposed closed mesocosm system suitable as a standard test?

Mesocosm system is suited to test disintegration

Disintegration of the three degradable test polymers PHB, PBSeT and PBSe occurred in all habitats in both years' experiments, although at different rates.

The differentiation by polymer was expected and is intrinsic to the material properties. The differentiation by the habitats beach, water column and seafloor is reasonable due to different biotic and abiotic conditions. The eulittoral samples were buried in the sediment, constantly in the dark and periodically immersed. In this habitat disintegration varied least, most likely because of the lower number of environmental factors (no light, no water current) and thus variables. In the water column irregular fouling, likely caused by inhomogeneous light and water movement regime, might have been responsible for variable disintegration rates. The same was observed for the tests at seafloor conditions, which might have been the most complex combination of environmental factors, with the samples exposed to two matrices at opposite sides.

The difference in disintegration rate in different replicate tanks is an unwanted fact, but might reflect the experimental drawback of not fully standardised matrices, i.e. natural sediments and seawater. The main environmental parameters as light, water movement, temperature, salinity and pH were controlled, and the sediments mixed before filling the three replicate test tank systems. However, microbial differences in the matrices in three independent closed-circuit tanks are very likely to diverge in the succession of microbial communities over time. This might even come down to the favoured or inhibited growth of specific microbial strains. In the eulittoral tests of year 1 PHB was much faster disintegrating in tank 1 at all 4 sampling intervals, whereas for PBSeT disintegration was similar in all three tanks (Figure 198). In the repetitive experiment of year 2 the disintegration of PHB, and also PBSeT and PBSe was similar in all three tanks for most of the samples. One of the methodical facts were the use of films of different thicknesses for different polymers. PHB films tested were 100 µm thick, whereas PBSe and PBSeT films were 25 µm thick. For standardised tests films of a common thickness with an intact surface (no wrinkles, cuts, tears) should be used.

The disintegration tests under eulittoral conditions revealed some interesting effects that could give hints to an optimisation towards a standardisation. The samples that were buried with a small inclination of only a few degrees from horizontal experienced a differentiation in disintegration likely according to the water cover during the applied tidal cycle. Also, in year 2 the sediment around some of the samples in the same tank was suspected to not fully draining leaving the samples longer or even permanently wet. This also may have resulted in an elevated disintegration rate. Unintentionally this technical variation introduced a fourth habitat, a permanently immersed sediment, in which the samples were completely buried.

The grain size, permeability, porosity, water retention capacity and water cover a sample and the different combination of these parameters could influence the disintegration rates, e.g. for PBSeT year 1 vs. year 2 (Figure 198).

To reduce high variations between replicates a mixture of different and pre-treated sediments could be used. Different sediment types, for example from three to five beaches could be collected and mixed. Then a pre-treatment should be applied, for example by vigorous aeration so that organics are turned over, and washing of the sediment so that fine particles are washed out. For comparability all experiments should use the same grain size fraction, selected by sieving.

Disintegration occurred at rather low rate

In our experiments, at the incubation temperature of 21 °C the disintegration in most samples from the year 1 experiments was below 50 % after 308 days. For a good part of the samples disintegration of year 2 was between 50 and 90 % after max. 271 days.

With respect to the laboratory tests which have been done at higher temperatures (up to 28 °C) it can be assumed that also in mesocosms the disintegration would be faster at higher temperatures. This would mean to mimic rather tropical than the temperate climate conditions we used in this study. This could be a useful modification to accelerate standard mesocosm tests for better practicability.

A further tool to increase the rate would be the addition of nutrients. In the tests reported here the concentrations of N- and P-compounds were low and might have slowed down the growth of microorganisms involved in the disintegration of the polymers. The addition could occur by regularly exchanging a part of the seawater and/or by adding KH_2PO_4, NH_4Cl and Fe fertilizer as it was done in the laboratory tests within this project (for details see Deliverable 5.7 Part 1).

The light conditions were reflecting low light conditions and when compared to the field they corresponded to depths of between 40 and 50 meters of water. Increasing the light would also increase the fouling by phototrophic organisms. If this would increase or slow down the disintegration rate is not clearly shown and further research is needed here.

PHB as a positive control

PHB was chosen as positive control instead of cellulose, which is often used in laboratory degradation experiments. Cellulose is not available as film rather than as a sheet of pressed fibres why it was expected to lose shape immediately, disintegrate and be washed out of the holding frame. PHB did show disintegration in all habitats in both years, however, the disintegration of PHB was faster than of PBSeT and PBSe only in the benthic tests (Figure 203). In the eulittoral and pelagic tests PHB disintegration was slow and only well measurable after longer exposure times. The PHB is not suited as a general positive control at 100 micron of thickness, but should be applied in thinner (25 µm) films to be able to measure changes already after shorter exposure times.

Measuring disintegration, not biodegradation

For mesocosm testing, the only standard available ASTM D7473-12 requires the determination of weight loss over time as a measure of disintegration. If weight loss is meas-

ured in fouled samples, i.e. with overgrowth of algae and animals, it is very difficult to tell apart the polymer weight from the adhering organisms. The removal of biofilm from the polymer before the measurement introduces a further possible source of error especially in case the sample is already very brittle or even fragmenting. For this reason it was decided to estimate the degree of disintegration by measuring the area of the scanned sample that has been lost during the experiment compared to the intact film at the start of the experiment as % area. Also this method has its drawback, as disintegration of a film is also a three-dimensional process in several phases. (1) the disintegration starts at the surface of the material, leading to a thinning. (2) The thinning of the film leads to small holes, later cracks and eventually to bigger holes and fragmentation. (3) At a progressed disintegration fragments will be lost from the samples. With the photogrammetrical measurement we regard the film as only a two-dimensional surface. This means a thinning is not measurable as long there are no visible holes formed and will lead to an underestimation of the disintegration at an early stage. This fact is well visible in the comparison of the data from PHB film with 100 µm with PBSeT or PBSe film of 25 µm thickness. The normalisation for thickness, as done here (Table 88 and Table 89), compensates for that, but might introduce other errors. Once holes and cracks are formed and a corrosive pattern is visible the measurement is quite accurate. An overestimation might occur at a very late stage of disintegration when fragments of still intact material detach from the sample and get lost. In the mesocosm experiments we successfully could prevent a significant loss of bigger fragments of the tested polymer films by covering them with a 4 x 4 mm mesh.

In open systems like the mesocosms it is not possible to directly measure the biodegradation of plastic, which is the complete conversion of the polymer into CO_2 (and/or CH_4), H_2O and biomass. Thus the polymers have to be tested first in closed laboratory tests for their inherent biodegradability and then checked in field or mesocosm tests for their behaviour in natural or more similar to natural conditions. Taking this into account a threshold or pass level for disintegration in a mesocosm test could be rather low, because it is an additional test in order to confirm the results of a previous laboratory test.

Generally none of the methods applied here to indirectly measure degradation as disintegration in an open system has given satisfactory results. Methods that regarded the molecular structure like MALDI-ToF and GPC did not show differences between visually disintegrated and non-treated materials. Tests of the mechanical properties could not be performed anymore from relatively slightly disintegrated samples. Thickness measurements were biased by fouling organisms. The photogrammetrical measurements are interesting because of their simplicity and could be optimized. Photographs should be made in a standardised set-up of the freshly retrieved samples and the whole test surface should be analysed in order to avoid time-consuming selection of the area of interest for each specimen. Test materials should be of the same thickness. Nevertheless, further research effort should be put also into the possible application of non-destructive methods as e.g. nano-CT or other techniques that could allow for the determination of the remaining polymer volume after the exposure, with distinction from fouling organisms. This would allow the measurement of disintegration of test materials at all stages of degradation.

5.2 Which test conditions are environmentally relevant?

In the global marine system typical temperatures range from polar -1.5 to tropical 30 °C. In the largest marine habitat, the deep-sea temperature is quite stable around 1 to 4 °C. Shallow tropical water is also quite stable between 25 and 30 °C. Temperate and sub-tropical regions experience seasonal fluctuations that can span over 20 °C or more at the surface.

Light conditions are very variable with geographic latitude and water depth. Illumination ranges from extreme solar radiation at very shallow depths in the tropics to complete darkness in the deep sea.

Most seawater is oxygenated to a certain extent but there can be local variations in the photic zone due to photosynthetic O_2 production by day which can even lead to an over-saturation, and O_2 consumption by night which in extreme can lead to a complete depletion. There are also oxygen minimum zones known in the world oceans where oxygen concentrations are always very low.

The pH of seawater is about 8.1 at the surface due to the exchange with the atmosphere and drops to about 7.5 in the deep sea. In eutrophicated waters pH might vary even more.

Sediment is a rather general term for the loose material found at the seafloor. Marine sediments range in grain size from pebbles at a high-energy beach to fine mud in sheltered bays or in the deep sea. Sandy sediments are permeable for water movement whereas in muddy sediments transport of substances is mainly by diffusion. Biologically shallow-water fine sediments often contain more organic substances and are more active than coarse sand.

To put this natural variety into a representative set of environmental conditions is selective and as a consequence neglects the majority of the ocean realm. The exclusion of light, a stable temperature of 20 – 25 °C, a salinity of 35 to 38, a pH of 8 and fine sand for the mesocosm tests are proposed. The use of muddy sediment is not feasible in the eulittoral test as performed here because mud will keep its porewater at low tide and will not drain at a falling water level. Mud could be used however for the benthic tests, but would represent a different habitat.

5.3 What does the heterogeneity between replicates tell us?

Natural environments are structured beyond our categorisation level. Each individual organism is participating in the structuring of its surrounding through metabolic activity as e.g. respiration, the formation of physical structures as e.g. body forms or mucus. The interaction of organisms with each other and the abiotic environment is resulting in a continuum of altering conditions and thus a mosaic of micro-habitats (Weber et al. 2015). Small scale differences of conditions right at the polymer sample surface most likely create microniches that are reflected in the heterogeneity of disintegration within one sample, within a time series and between replicates although similar abiotic conditions like salinity, pH, oxygen, temperature and light are maintained within narrow ranges. One parameter that seemed to have a strong structuring effect is light, which is also difficult to standardise in a mesocosm system. That is why it is recommended to exclude light from mesocosm experiments.

5.4 Which marine habitats are missing?

The combination of three habitats with two chemically different sediments in a connected test system with a common water circuit was successfully kept stable for more than 10 months, by adjusting only salinity with distilled water to compensate for evaporation loss. Thus a technically rather simple and robust test system is made available and can be applied independently from the direct supply of running seawater in any laboratory. In addition to the tidal beach scenario in the eulittoral test tank a fourth habitat could be added. If the eulittoral test tank is filled with a thicker sediment layer and the draining of the percolated water is allowed only to the layer higher than 10 cm another set of samples could be buried in the sand. This way the scenario of a polymer being buried in a completely immersed permanently wet sediment cold be mimicked in the existing system. Different sediment types occur in the marine environment. Especially mud plays an import role. In coastal areas muddy sediment is found in accumulation spaces as enclosed bays, estuaries and harbours. These areas naturally accumulate organic particles and are also at high risk as a sink for anthropogenic waste especially plastic litter. Half of our planet is covered by deep-sea mud which is very low in organics and nutrients. The deep sea has also been identified as the major sink of marine plastic litter. In order to cover more representative marine habitats shallow-water, organic-rich mud and deep-sea mud should be taken into account for further testing.

In the mesocosm experiments oxygen was present at all times. Within fine sediments and in parts of the world's oceans however there is little or no oxygen available, especially within sediments. Tests under hypoxic and anoxic conditions are needed to assess the fate of plastic in these habitats. Ideally more information on the effect of temperature on the degradation/disintegration is gained by repeating the mesocosm experiments under a variety of environmentally relevant temperatures, i.e. from 0 to 30 °C, oxygen concentrations and sediment types (mud to coarse sand).

Another experimentally challenge is faced if the conditions of half of the world's ocean, namely the deep sea are to be tested. Pressures of 37 Mpa (370 atmospheres) that correspond to the average deep-sea depth of 3700 m are difficult to achieve in laboratory or even mesocosm tests. *In-situ* field test seems to be the more feasible alternative to include this important habitat.

6 Conclusion

This work within work package 5 is linked to the laboratory tests (Deliverable 5.7 part 1) and tests in the field (Deliverable 5.8) and should provide information if the here developed mesocosm system can be proposed for the development of a standard test. Based on the successfully performed tests presented here, it was concluded that the developed closed test system for aerobic conditions works well for the assessment of the disintegration of plastic under marine conditions. Further modifications are recommended to achieve faster disintegration rates, and to reduce heterogeneity within replicates. Based on the data presented it was assumed that this should be able by increasing the temperature, excluding light, adding nutrients, and using a mixture of sediment. However, these modifications need to be tested and evaluated systematically. Further, one has to be aware that these tests represent a selection from all existing marine habitats. In this study only three relevant oxygenated shallow-water habitats were tested.

Another conclusion was that the methods used to analyse polymer degradation (physical: tensile properties determination; chemical: GPC and MALDI-TOF techniques) were not suitable for this test. Further investigations towards a reliable measure that allow to link the observed disintegration to changing material properties during degradation would be useful.

7 Acknowledgements

We thank the Parco Nazionale Arcipelago Toscano, Portoferraio, Isola d'Elba, Italy for granting access to the protected area of Isola di Pianosa with the research permit n.3063/19.05.2014.

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9 Appendix

Appendix table 1: Characterisation of the seawater of the pelagic and benthic habitat used during the mesocosom experiment year 1.

Pelagic / benthic				tank 1						tank 2						tank 3					
				interval						interval						interval					
Nutrient-related parameters	unit	detection limit	t_0	t_1	t_2	t_3	t_4	MV	SD	t_1	t_2	t_3	t_4	MV	SD	t_1	t_2	t_3	t_4	MV	SD
Nitrogen (total-N; TN)	mg/L	0.25	**1.20**	**0.90**	**0.50**	**0.44**	**0.45**	0.57	0.22	**0.61**	**0.30**	**0.38**	**0.26**	0.39	0.16	**0.63**	**0.40**	**0.41**	**0.29**	0.43	0.14
Total Inorg. Nitrogen (TIN)	mg/L	calculated	**0.69**	**0.13**	**0.18**	**0.04**	<0.08	0.12	0.07	**0.05**	**0.08**	**0.03**	<0.08	0.05	0.03	**0.05**	**0.10**	**0.03**	**0.03**	0.05	0.03
Total Org. Nitrogen (TON)	mg/L	calculated	**0.51**	**0.77**	**0.32**	**0.40**	**0.45**	0.49	0.20	**0.57**	**0.22**	**0.35**	**0.26**	0.35	0.15	**0.58**	**0.30**	**0.39**	**0.27**	0.38	0.14
Ammonium (NH_4-N)	mg/L	0.04	**0.18**	<0.04	0.05	<0.04	<0.04	0.05		<0.04	<0.04	<0.04	<0.04			<0.04	<0.04	<0.04	<0.04		
Nitrite (NO^2-N)	mg/L	0.02	**0.04**	<0.02	**0.13**	<0.02	<0.02	0.13		<0.02	**0.08**	<0.02	<0.02	0.08		<0.02	**0.10**	<0.02	<0.02	0.10	
Nitrate (NO^3-N)	mg/L	0.02	**0.47**	**0.13**	<0.02	**0.04**	<0.02	0.09	0.06	**0.05**	<0.02	**0.03**	<0.02	0.04	0.01	**0.05**	<0.02	**0.03**	**0.03**	0.04	0.01
Phosphor (total-P)	mg/L	0.01	**0.02**	**0.02**	**0.02**	**0.01**	**0.08**	0.03	0.03	**0.02**	**0.01**	**0.01**	**0.08**	0.03	0.03	**0.01**	**0.02**	**0.01**	**0.07**	0.03	0.03
Ortho-Phosphate (PO_4-P)	mg/L	0.01 resp. 0.005	<0.01	<0.01	<0.01	**0.01**	0.01	0.01	0.00	<0.01	<0.01	<0.005	<0.005			**0.01**	<0.01	<0.005	**0.006**	0.01	
Dissolved Org. P (DOP)	mg/L	calculated	**0.02**	**0.02**	**0.02**	**0.00**	**0.07**	0.03	0.03	**0.02**	**0.01**	**0.01**	**0.08**	0.03	0.03	**0.00**	**0.02**	**0.01**	**0.07**	0.02	0.03
Total Carbon (TC)	mg/L	1	**36**	**16**	**34**	**28**	**32**	28	8	**15**	**59**	**29**	**39**	36	19	**13**	**37**	**35**	**35**	30	11
Total Inorg. Carbon (TIC)	mg/L	-	**n.a.**	**n.a.**	**n.a.**	**26**	**27**	26	1	**n.a.**	**n.a.**	**27**	**32**	30	4	**n.a.**	**n.a.**	**30**	**30**	30	0
Total Org. Carbon (TOC)	mg/L	1	**n.a.**	**n.a.**	**8.60**	**2.40**	**5.50**	5.50	3.10	**n.a.**	**3.0**	**2.0**	**6.7**	3.9	2.5	**n.a.**	**2.7**	**4.6**	**4.9**	4.1	1.2
Dissolved Org. Carbon (DOC)	mg/L	1	**3.10**	**2.90**	**2.60**	**2.00**	**5.50**	3.25	1.55	**3.0**	**2.5**	**1.9**	**3.2**	2.7	0.6	**3.1**	**2.6**	**2.6**	**3.5**	3.0	0.4
Particulate Org. Carbon (POC)	mg/L	calculated	**n.a.**	**n.a.**	**6.00**	**0.40**	**0.00**	2.13	3.35	**n.a.**	**0.5**	**0.1**	**3.5**	1.4	1.9	**n.a.**	**0.1**	**2.0**	**1.4**	1.2	1.0
Chlorophyll *a*	µg/l	1 resp. 0.01	<1	**1.80**	**0.60**	<1	<1	1.20	0.85	<1	**0.3**	<1	<1	0.3		**3.6**	**0.6**	<1	<1	2.1	2.1
Phaeopigment	µg/l	1	**1.50**	**4.00**	**0.90**	**2.30**	<1	2.40	1.55	**2.9**	**0.5**	<1	**1.4**	1.6	1.2	**3.1**	**0.7**	**1.3**	<1	1.7	1.2
Silica (as SiO_2)	mg/L	2 resp. 0.4	<2	<2	**0.53**	<0.4	<0.4	0.53		<2.0	**0.47**	<0.4	<0.4	0.47		<2.0	**0.36**	<0.4	<0.4	0.36	
Anions and metals																					
Iron	mg/L	0.1	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	<0.1	<0.1	<0.1			<0.1	**0.2**	<0.1	<0.1	0.20	
Manganese	mg/L	0.05	<0.05	<0.05	<0.05	<0.05	<0.05			<0.05	<0.05	<0.05	<0.05			<0.05	<0.05	<0.05	<0.05		
Aluminium	mg/L	0.05	**0.09**	**0.11**	**0.11**	<0.05	**0.09**	0.10	0.01	**0.12**	**0.11**	<0.05	**0.10**	0.11	0.01	**0.13**	**0.11**	<0.05	**0.07**	0.10	0.03

sampling dates: t_0 22.09.14; t_1 10.12.14; t_2 18.02.15; t_3 05.05.15; t_4 28.07.15

n.a. = not available

Appendix table 2: Characterisation of the seawater of the pelagic and benthic habitat used during the mesocosom experiment year 2.

Pelagic / benthic			tank 1						tank 2						tank 3					
			interval						interval						interval					
Nutrient-related parameters	unit	detection limit	$t_{2.1}$	$t_{2.2}$	$t_{2.3}$	$t_{2.4}$	MV	SD	$t_{2.1}$	$t_{2.2}$	$t_{2.3}$	$t_{2.4}$	MV	SD	$t_{2.1}$	$t_{2.2}$	$t_{2.3}$	$t_{2.4}$	MV	SD
Nitrogen (total-N; TN)	mg/L	0.25	**0.78**	**0.63**	<0.25	**0.26**	0.56	0.27	**0.74**	**0.70**	<0.25	**0.25**	0.56	0.27	**0.87**	**0.50**	<0.25	**0.25**	0.54	0.31
Total Inorg. Nitrogen (TIN)	mg/L	calculated	**0.11**	**0.03**	<0.04	<0.04	0.07	0.05	**0.12**	**0.03**	**0.06**	<0.04	0.07	0.05	**0.14**	**0.03**	**1.39**	<0.04	0.52	0.76
Total Org. Nitrogen (TON)	mg/L	calculated	**0.67**	**0.60**	<0.25	<0.25	0.64	0.05	**0.62**	**0.68**	<0.25	<0.25	0.65	0.04	**0.73**	**0.47**	<0.25	<0.25	0.60	0.19
Ammonium (NH_4-N)	mg/L	0.04	**0.08**	<0.04	<0.04	<0.04	0.08		**0.07**	<0.04	**0.06**	<0.04	0.06	0.01	**0.08**	<0.04	1.30	<0.04	0.69	0.87
Nitrite (NO^2-N)	mg/L	0.02	<0.02	<0.02	<0.02	<0.02			<0.02	<0.02	<0.02	<0.02			<0.02	<0.02	<0.02	<0.02		
Nitrate (NO^3-N)	mg/L	0.02	**0.03**	**0.03**	<0.02	<0.02	0.03	0.00	**0.05**	**0.03**	<0.02	<0.02	0.04	0.02	**0.06**	**0.03**	**0.09**	<0.02	0.06	0.03
Phosphor (total-P)	mg/L	0.01	**0.01**	**0.03**	**0.01**	**0.02**	0.02	0.01	**0.01**	**0.03**	**0.05**	**0.02**	0.03	0.02	**0.01**	**0.02**	**0.11**	**0.02**	0.04	0.05
Ortho-Phosphate (PO_4-P)	mg/L	0.01 resp. 0.005	<0.005	**0.01**	**0.01**	**0.01**	0.01	0.00	<0.005	**0.01**	**0.04**	**0.01**	0.02	0.02	<0.005	<0.005	<0.25	**0.01**	0.01	
Dissolved Org. P (DOP)	mg/L	calculated	<0.010	<0.005	<0.005	<0.005			<0.007	**0.01**	<0.005	<0.005	0.0		<0.006	<0.005	<0.005	<0.005		
Total Carbon (TC)	mg/L	1	**38**	**39**	**40**	**38**	39	1	**39**	**42**	**40**	**32**	38	4	**38**	**33**	**40**	**37**	37	3
Total Inorg. Carbon (TIC)	mg/L	-	**33**	**34**	**38**	**35**	35	2	**36**	**37**	**37**	**29**	35	4	**33**	**30**	**37**	**34**	34	3
Total Org. Carbon (TOC)	mg/L	1	**4.8**	**4.9**	**2.4**	**2.7**	3.7	1.3	**3.2**	**2.9**	**3.5**	**2.7**	3.1	0.4	**5.1**	**3.9**	**3.0**	**3.0**	3.8	1.0
Dissolved Org. Carbon (DOC)	mg/L	1	**3.6**	**3.9**	**2.4**	**2.3**	3.1	0.8	**2.7**	**3.9**	**1.8**	**2.3**	2.7	0.9	**3.5**	**2.2**	**2.3**	**1.9**	2.5	0.7
Particulate Org. Carbon (POC)	mg/L	calculated	**1.2**	**0.7**	**0.0**	**0.4**	0.6	0.5	**0.5**	**1.0**	**1.7**	**0.4**	0.9	0.6	**1.6**	**0.7**	**0.7**	**1.1**	1.0	0.4
Chlorophyll *a*	µg/l	1 resp. 0.01	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Phaeopigment	µg/l	1	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Silica (as SiO_2)	mg/L	2 resp. 0.4	<0.4	<0.4	n.a.	n.a.			**0.40**	<0.4	n.a.	n.a.	0.40		<0.4	<0.4	n.a.	n.a.		
Anions and metals																				
Iron	mg/L	0.1	<0.1	<0.1	<0.1	<0.1			<0.1	<0.1	<0.1	<0.1			<0.1	<0.1	<0.1	<0.1		
Manganese	mg/L	0.05	<0.05	<0.05	<0.05	<0.05			<0.05	<0.05	<0.05	<0.05			<0.05	<0.05	<0.05	<0.05		
Aluminium	mg/L	0.05	**0.23**	<0.05	<0.05	<0.05	0.23		**0.22**	<0.05	<0.05	<0.05	0.22		**0.21**	<0.05	<0.05	<0.05	0.21	

sampling dates: $t_{2.1}$ 15.12.15; $t_{2.2}$ 26.02.16; $t_{2.3}$ 23.05.16; $t_{2.4}$ 25.06.15

n.a. = not available

Appendix table 3: Characterisation of the seawater of the eulittoral habitat used during the mesocosom experiment year 1.

Mesocosm year 1				tank 1						tank 2						tank 3					
Pelagic / benthic				interval						interval						interval					
Nutrient-related parameters	unit	detection limit	t_0	t_1	t_2	t_3	t_4	MV	SD	t_1	t_2	t_3	t_4	MV	SD	t_1	t_2	t_3	t_4	MV	SD
Nitrogen (total-N; TN)	mg/L	0.25	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Total Inorg. Nitrogen (TIN)	mg/L	calculated	**0.64**	**0.15**	**0.11**	**0.04**	<0.08	0.10	0.06	**0.05**	**0.08**	**0.04**	<0.08	0.06	0.02	**0.06**	**0.16**	**0.02**	**0.05**	0.07	0.06
Total Org. Nitrogen (TON)	mg/L	calculated	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Ammonium (NH_4-N)	mg/L	0.04	**0.18**	<0.04	<0.04	<0.04	<0.04			<0.04	<0.04	<0.040	<0.04			<0.04	**0.07**	<0.04	<0.04	0.07	
Nitrite (NO^2-N)	mg/L	0.02	**0.04**	<0.02	**0.11**	<0.02	<0.02	0.11		<0.02	**0.08**	<0.020	<0.02	0.08		<0.02	**0.09**	<0.02	<0.02	0.09	
Nitrate (NO^3-N)	mg/L	0.02	**0.42**	**0.15**	<0.02	**0.04**	<0.02	0.10	0.08	**0.05**	<0.02	**0.04**	<0.02	0.05	0.01	**0.06**	<0.02	**0.02**	**0.05**	0.05	0.02
Phosphor (total-P)	mg/L	0.01	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Ortho-Phosphate (PO_4-P)	mg/L	0.01 resp. 0.005	<0.01	<0.01	<0.01	<0.005	<0.005			<0.01	<0.01	<0.005	**0.01**	0.01		<0.01	<0.01	**0.01**	**0.01**	0.01	0.00
Dissolved Org. P (DOP)	mg/L	calculated	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Total Carbon (TC)	mg/L	1	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Total Inorg. Carbon (TIC)	mg/L	-	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Total Org. Carbon (TOC)	mg/L	1	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Dissolved Org. Carbon (DOC)	mg/L	1	**3.8**	**3.6**	**5.8**	**2.3**	**6.8**	4.6	2.0	**4.0**	**8.1**	**3.1**	**3.9**	4.8	2.3	**3.6**	**11.0**	**2.4**	**6.1**	5.8	3.8
Particulate Org. Carbon (POC)	mg/L	calculated	n.a.	n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.			n.a.	n.a.	n.a.	n.a.		
Chlorophyll *a*	µg/l	1 resp. 0.01	<1	n.a.	**1.0**	**3.9**	n.a.	2.5	2.1	n.a.	<0.1	<1.0	**2.0**	2.0		n.a.	**2.0**	<1.0	**1.8**	1.9	0.2
Phaeopigment	µg/l	1	<1	n.a.	**3.9**	**5.1**	n.a.	4.5	0.8	n.a.	**4.9**	**6.5**	**3.6**	5.0	1.5	n.a.	**2.2**	**9.7**	**5.8**	5.9	3.8
Silica (as SiO_2)	mg/L	2 resp. 0.4	<2	<2	**0.75**	<0.4	<0.4	0.75		<2.0	**0.45**	<0.4	<0.5	0.45		<2.0	**0.57**	<0.4	**0.51**	0.54	0.04

sampling dates: t_0 22.09.14; t_1 10.12.14; t_2 18.02.15; t_3 06.05.15; t_4 25.07.15
n.a. = not available

Appendix table 4: Heavy metals of the seawater of each tank of the pelagic/benthic test and in the porewater of the eulittoral habitat during the mesocosom experiment year 1. The analytical methods were done according to the mentioned standard.

			habitat	**pelagic / benthic**			**eulittoral**		
			date	05.05.15	05.05.15	05.05.15	06.05.15	06.05.15	06.05.15
Heavy metals	**Number of standard**	unit	detection limit	tank 1	tank 2	tank 3	tank 1	tank 2	tank 3
Lead	ISO 11885-E22:2009-09	mg/L	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cadmium	ISO 11885-E22:2009-09	mg/L	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Zinc	ISO 11885-E22:2009-09	mg/L	0.01	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Copper	ISO 11885-E22:2009-09	mg/L	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mercury	ISO 12846-E12:2012-08	mg/L	0.0005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005
Nickel	ISO 11885-E22:2009-09	mg/L	0.01	<0.01	<0.01	<0.01	<0.01	n.a.	n.a.
Arsenic	ISO 11885-E22:2009-09	mg/L	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Chromium	ISO 11885-E22:2009-09	mg/L	0.01	<0.01	<0.01	<0.01	n.a.	n.a.	n.a.

n.a. = not available

Appendix table 5: Characterisation of the sediment used for the eulittoral tests during the mesocosom experiment year 1 and 2.

Eulittoral		Sampling date	22.09.14	08.05.15	08.05.15	08.05.15			05.09.15	05.09.15	05.09.15		
Physical parameters	unit	detection limit	t_0	tank 1	tank 2	tank 3	MV	SD	tank 1	tank 2	tank 3	MV	SD
Dry weight	% w/w		**82**	**75**	**74**	**76**	75	1	**75**	**74**	**76**	75	1
Ignition loss @ 550°C	% dw		**1**	**1**	**1**	**1**	1	0	**0.8**	**0.8**	**0.7**	1	0
Ignition residue @ 800°C	% dw		**99**	**99**	**99**	**99**	99	0	**99**	**99**	**99**	99	0
Permeability	m^2		n.a.	**2.4110^{-10} ± $6.66*10^{-11}$ ***	**1.5410^{-11} ± 4.8710^{-12} ***	**1.4210^{-11} ± 2.7310^{-11} ***	1.7910^{-10}	5.8210^{-12}	**8.4410^{-11} ± 6.2210^{-12}**	**1.1010^{-10} ± 8.2410^{-12}**	**9.1610^{-11} ± 4.0110^{-11}**	9.5410^{-11}	2.2010^{-11}
Porosity	% v/v		n.a.	**45.25 ± 1.75 ***	**42.71 ± 0.27 ***	**45.99 ± 0.32 ***	45	2	**37.09 ± 0.72**	**49.41 ± 10.21**	**39.00 ± 3.12**	42	8
Grain size (median)	µm		n.a.	**269 ± 21 ***	**273 ± 5 ***	**290 ± 5 ***	278	14	**212 ± 5**	**194 ± 2**	**212 ± 1**	206	9
Nutrient-related parameters													
Nitrogen (total-N; TN)	mg/kg	1000	<1000	<1000	<1000	<1000	<1000	0	<1000	<1000	<1000	<1000	0
Phosphorus (total-P)	mg/kg	20	30	**55**	**52**	**57**	55	3	**55**	**52**	**57**	55	3
Total Carbon (TC)	% dw	0.5 resp. 0.2	<0.5	<0.2	<0.2	<0.2	<0.2	0	<0.2	<0.2	<0.2	<0.2	0
Total Organic Carbon (TOC)	% dw	0.5 resp. 0.2	<0.5	<0.2	<0.2	<0.2	<0.2	0	<0.2	<0.2	<0.2	<0.2	0
Metals and metalloids													
Aluminium	mg/kg	100	1700	**3400**	**3200**	**3300**	3300	100	**3400**	**3200**	**3300**	3300	100
Sulfur	mg/kg	100	n.a.	**350**	**340**	**310**	333	21	**350**	**340**	**310**	333	21
Lead	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Cadmium	mg/kg	0.5	n.a.	<0.5	<0.5	<0.5	<0.5	0	<0.5	<0.5	<0.5	<0.5	0
Zinc	mg/kg	10	n.a.	<10	<10	<10	<10	0	<10	<10	<10	<10	0
Copper	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Mercury	mg/kg	0.05	n.a.	<0.05	<0.05	<0.05	<0.05	0	<0.05	<0.05	<0.05	<0.05	0
Nickel	mg/kg	5	n.a.	**9**	**9**	**9**	9	0	**9**	**9**	**9**	9	0
Arsenic	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Chromium	mg/kg	5	n.a.	**34**	**35**	**29**	33	3	**34**	**35**	**29**	33	3
Magnesium	mg/kg	100	1200	**2200**	**2100**	**2100**	2133	58	**2200**	**2100**	**2100**	2133	58
Potassium	mg/kg	100	600	**1400**	**1200**	**1300**	1300	100	**1400**	**1200**	**1300**	1300	100

Sodium	mg/kg	100	1700	**4200**	**4100**	**3500**	3933	379	**4200**	**4100**	**3500**	3933	379
Calcium	mg/kg	100	1500	**3200**	**3200**	**3500**	3300	173	**3200**	**3200**	**3500**	3300	173
Iron	mg/kg	100	1350	**2400**	**2300**	**2400**	2367	58	**2400**	**2300**	**2400**	2367	58
Manganese	mg/kg	100	<100	<100	<100	<100	<100	0	<100	<100	<100	<100	0

*sampling date: 05.08.15; n.a.: not available

Appendix table 6: Characterisation of the sediment used for the benthic tests during the mesocosom experiment year 1 and 2.

Benthic		Sampling date	22.09.14	09.05.15	09.05.15	09.05.15			05.08.15	05.08.15	05.08.15		
Physical parameters	unit	detection limit	t_0	tank 1	tank 2	tank 3	MV	SD	tank 1	tank 2	tank 3	MV	SD
Dry weight	% w/w		**67**	**56**	**54**	**58**	56	2	**56**	**54**	**58**	56	2
Ignition loss @ 550°C	% dw		**5**	**6**	**6**	**6**	6	0	**6**	**6**	**6**	6	0
Ignition residue @ 800°C	% dw		**59**	**57**	**57**	**57**	57	0	**57**	**57**	**57**	57	0
Permeability	m^2		n.a.	**$2.60*10^{-11} \pm 1.29*10^{-11}$ ***	**$2.51*10^{-11} \pm 1.21*10^{-12}$ ***	**$2.69*10^{-11} \pm 5.17*10^{-12}$ ***	$2.60 *10^{-11}$	$6.28 *10^{-12}$	**$1.52*10^{-11} \pm 7.537*10^{-12}$**	**$2.83*10^{-11} \pm 1.98*10^{-11}$**	**$2.87*10^{-11} \pm 6.81*10^{-12}$**	$2.40 *10^{-11}$	$1.21 *10^{-11}$
Porosity	% v/v		n.a.	**64.31 ± 4.24 ***	**58.79 ± 1.62 ***	**64.33 ± 1.74 ***	62	4	**49.03 ± 8.59**	**48.21 ± 2.26**	**54.58 ± 8.84**	51	6
Grain size (median)	µm		n.a.	**187 ± 7 ***	**189 ± 28 ***	**168 ± 35 ***	181	23	**129 ± 41**	**147 ± 60**	**161 ± 69**	146	47
Nutrient-related parameters													
Nitrogen (total-N; TN)	mg/kg	1000	<1000	<1000	<1000	<1000	<1000	0	<1000	<1000	<1000	<1000	0
Phosphorus (total-P)	mg/kg	20	**100**	**200**	**190**	**190**	193	6	**200**	**190**	**190**	193	6
Total Carbon (TC)	% dw	0.5 resp. 0.2	**12**	**12**	**12**	**12**	12	0	**12**	**12**	**12**	12	0
Total Organic Carbon (TOC)	% dw	0.5 resp. 0.2	n.a.	**0.29**	**0.31**	**0.29**	0	0	**0.29**	**0.31**	**0.29**	0	0
Metals and metal-loids													
Aluminium	mg/kg	100	**710**	**1100**	**1100**	**1100**	1100	0	**1100**	**1100**	**1100**	1100	0
Sulfur	mg/kg	100	n.a.	**2700**	**2800**	**2600**	2700	100	**2700**	**2800**	**2600**	2700	100
Lead	mg/kg	5	n.a.	**6**	**5**	**5**	5	1	**6**	**5**	**5**	5	1
Cadmium	mg/kg	0.5	n.a.	<0.5	<0.5	<0.5	<0.5	0	<0.5	<0.5	<0.5	<0.5	0
Zinc	mg/kg	10	n.a.	<10	<10	<10	<10	0	<10	<10	<10	<10	0
Copper	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Mercury	mg/kg	0.05	n.a.	<0.05	<0.05	<0.05	<0.05	0	<0.05	<0.05	<0.05	<0.05	0
Nickel	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Arsenic	mg/kg	5	n.a.	<5	<5	<5	<5	0	<5	<5	<5	<5	0
Chromium	mg/kg	5	n.a.	**6**	**5**	**5**	5	1	6	5	5	5	1

Magnesium	mg/kg	100	**9600**	**20200**	**18300**	**19000**	19167	961	**20200**	**18300**	**19000**	19167	961
Potassium	mg/kg	100	n.a.	**700**	**700**	**600**	667	58	**700**	**700**	**600**	667	58
Sodium	mg/kg	100	n.a.	**11700**	**12300**	**11300**	11767	503	**11700**	**12300**	**11300**	11767	503
Calcium	mg/kg	100	**329000**	**343000**	**319000**	**331000**	331000	12000	**343000**	**319000**	**331000**	331000	12000
Iron	mg/kg	100	n.a.	**1200**	**1100**	**1100**	1133	58	**1200**	**1100**	**1100**	1133	58
Manganese	mg/kg	100	n.a.	**120**	**110**	**110**	113	6	**120**	**110**	**110**	113	6

*sampling date: 04.08.15; n.a.: not available

Appendix table 7: Standards used for the analysis of the water and sediments used in the mesocosm experiments.

Water		Sediment			
Nutrient-related parameters	**Number of standard**	**Physical parameters**	**Number of standard**	**Metals and metalloids**	**Number of standard**
Nitrogen (total-N; TN)	EN 12260-H34:2003-12	Dry weight	ISO 11465/EN 14346	Aluminium	ISO 11885-E22:2009-07
Total Inorg. Nitrogen (TIN)	calculated	Ignition loss @ 550°C	DIN 38414-S3/EN 15169	Sulfur	ISO 11885-E22:2009-09
Total Org. Nitrogen (TON)	calculated	Ignition residue @ 800°C	DIN 38414-S3:1985-11	Lead	ISO 11885-E22:2009-07
Ammonium (NH_4-N)	ISO 11732-E23:2005-05	Permeability	Analysed by HYDRA	Cadmium	ISO 11885-E22:2009-07
Nitrite (NO^2-N)	ISO 13395-D28:1996-12	Porosity	Analysed by HYDRA	Zinc	ISO 11885-E22:2009-07
Nitrate (NO^3-N)	ISO 13395-D28:1996-12	Grain size (median)	Analysed by HYDRA	Copper	ISO 11885-E22:2009-07
Phosphor (total-P)	ISO 6878-D11:2004-09	**Nutrient-related parameters**		Mercury	ISO 16772:2005-06
Ortho-Phosphate (PO_4-P)	ISO 6878-D11:2004-09	Nitrogen (total-N; TN)	ISO 11261:1997-05	Nickel	ISO 11885-E22:2009-07
Dissolved Org. P (DOP)	calculated	Phosphorus (total-P)	ISO 11885-E22:2009-07	Arsenic	ISO 11885-E22:2009-07
Total Carbon (TC)	EN 1484-H3:1997-08	Total Carbon (TC)	ISO 10694:1996-08	Chromium	ISO 11885-E22:2009-07
Total Inorg. Carbon (TIC)	calculated	Total Organic Carbon (TOC)	ISO 10694:1996-08	Magnesium	ISO 11885-E22:2009-07
Total Org. Carbon (TOC)	EN 1484-H3:1997-08			Potassium	ISO 11885-E22:2009-07
Dissolved Org. Carbon (DOC)	EN 1484-H3:1997-08			Sodium	ISO 11885-E22:2009-07
Particulate Org. Carbon (POC)	calculated			Calcium	ISO 11885-E22:2009-07
Chlorophyll *a*	DIN 38412-L16:1985-12			Iron	ISO 11885-E22:2009-07
Phaeopigment	DIN 38412-L16:1985-12			Manganese	ISO 11885-E22:2009-07
Silica (as SiO_2)	DIN 38405-D21:1990-10				
Anions and metals					
Iron	ISO 11885-E22:2009-09				
Manganese	ISO 11885-E22:2009-09				
Aluminium	ISO 11885-E22:2009-09				

Appendix table 8: Organotin compounds and pesticides in combined samples from all three tanks of the seawater of the pelagic/benthic habitat and the porewater of the benthic and the eulittoral tests during the mesocosom experiment year 1. The analyses were done according to the mentioned standards.

			habitat	pelagic	benthic	eulittoral
			sampling date	05.05.15	14.05.15	07.05.15
Organotin compounds	**standard**	unit	detection limit	tank 1-3	tank 1-3	tank 1-3
Monobutyltin	DIN EN ISO 1735331:2015-11	µg/l	1	<1	<1	<1
Dibutyltin	DIN EN ISO 1735331:2015-11	µg/l	0.1	<0.01	<0.01	<0.001
Tributyltin	DIN EN ISO 1735331:2015-11	µg/l	0.001	<0.001	<0.001	<0.001
Tetrabutyltin	DIN EN ISO 1735331:2015-11	µg/l	0.001	<0.001	<0.001	<0.001
Tricyclohexyltin cation	DIN EN ISO 1735331:2015-11	µg/l	0.001	<0.001	<0.001	<0.001
Triphenyltin cation	DIN EN ISO 1735331:2015-11	µg/l	0.001	<0.001	<0.001	<0.001
Pesticides						
AMPA	ISO 21458:2008E	µg/l	0.025	<0.025	<0.025	<0.025
Atrazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Bentazon	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Bentazon-6OH	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Bentazon-8OH	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Bromacil	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chloridazon-desphenyl (B)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chloridazon-methyl-desphenyl (B1)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chlortoluron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Desethyl-Atrazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Desethylterbutylazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Desisoprpoylatrazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Dicamba	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Dichlorprop (Racemat)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
2.6-Dichlorbenzamid	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
N.N-Dimethylsulfamid (DMS)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Dimethachlor-Sulfonsäure (CGA 354742)	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Dimethachlor-Metabolit (CGA 369873)	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Dimethachlorsäure (CGA 50266)	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Diuron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025

Ethidimuron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Ethofumesat	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Glyphosat	ISO 21458:2008E	µg/l	0.025	<0.025	<0.025	<0.025
Isoproturon	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Mecoprop (Racemat)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metalaxyl (Racemat)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metamitron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metazachlor	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metazachlorsäure (BH 479-4)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metazachlorsulfonsäure (BH 479-8)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metolachlor (Racemat CGA 77101/CGA 77102)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metolachlorsäure (Racemat CGA 51202/CGA 351916)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metolachlor-Sulfonsäure (Racemat CGA 380168/CGA 354743)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metolachlor-Sulfonsäure (NOA 413173)	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metoxuron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Metribuzin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Oxadixyl	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Simazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Terbuthylazin	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Prothioconazol	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Bromoxynil	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chlofenvinphos	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chloridazon	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Chlorpyrifos (Chlorpyrifosethyl)	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	<0.025	<0.025	<0.025
Chlorpyrifosmethyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
Diflufenican	DIN 38407 F36: 2014-09	µg/l	0.025	n.a.	n.a.	n.a.
Methabenzthiazuron	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
MCPA	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Primicarb	DIN 38407 F36: 2014-09	µg/l	0.025	<0.025	<0.025	<0.025
Trifluralin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	<0.025	<0.025	<0.025
DDX und HCH	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
o-p DDD	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.

p-p DDD	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
o-p DDE	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
o-p DDT	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
p-p DDT	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	<0.025	<0.025	<0.025
alpha-HCH	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
beta-HCH	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
gamma-HCH (Lindan)	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	<0.025	<0.025	<0.025
delta-HCH (Lindan)	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.025	n.a.	n.a.	n.a.
Acetamiprid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Ametroctadin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Amidosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Azoxystrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Beflubutamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Benalaxyl-M	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Benthiavalicarb-isopropyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Bixafen	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Boscalid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Carbendazim	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Carfentrazon-ethyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Clethodim	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Clodinafop-propagyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Clomazone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Clothianidin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Cyazofamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Cycloxydim	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Cyproconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Cyprodinil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Difenconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Diflufenican	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Dimethachlor	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Dimethenamid-P	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Dimethoate	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Dimethomorph	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Dimoxystrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005

Epoxiconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenamidone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenazaquin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenhexamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenoxaprop-P	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenoxycarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenpropidin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fenpyroxymat	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flazasulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flonicamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Florasulam	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fluazifop-P	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fludioxonil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flufenacet	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fluopicolide	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fluoxastrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flupyrsulfuron-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fluroxypyr-methylhepthyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flurtamone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flusilazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Flutolanil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Foramsulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fosthiazat	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Fuberidazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Haloxyfop-P	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Hexythiazox	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Imazalil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Imazosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Imidacloprid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Indoxacarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Ioxynil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Iprovalicarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Isoxaben	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Lenacil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Mandipropamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005

Mepanipyrim	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Mesosulfuron-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Mesotrione	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Metalaxyl-M	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Metconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Methiocarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Metosulam	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Metrafenone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Metsulfuron-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Myclobutanil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Napropamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Nicosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Paclobutrazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Penconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pencycuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pethoxamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Phenmedipham	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Picolinafen	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Picoxystrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pinoxaden	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pirimiphos-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Prochloraz	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Propamocarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Propaquizafop	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Propiconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Propoxycarbazone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Propyzamid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Proquinazid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Prosulfocarb	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Prosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pymetrozin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pyraclostrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pyraflufen-ethyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pyrimethanil	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Pyroxsulam	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005

Quinmerac	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Quinoclamin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Quizalofop-P	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Silthiopham	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Sulcotrione	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Sulfosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Tebuconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Tembotrione	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Tepraloxidim	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Tetraconazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thiabendazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thiacloprid	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thiamethoxam	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thiencarbazone-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thifensulfuron-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Thiophanat-methyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Topramezone	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Triasulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Triclopyr-2-buthoxyethyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Trifloxystrobin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Triflusulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Triticonazol	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Tritosulfuron	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Warfarin	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Zoxamide	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Acequinocyl	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Aclonifen	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Aldrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Bifenox	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Bromophos-ethyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Captan	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Chlorpropham	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Chlorpyrifos-ethyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Chlorpyrifos-methyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005

Chlorthalonil	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Cyfluthrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Cyhalothrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Cypermethrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDD o.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDD p.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDE o.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDE p.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDT o.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
DDT p.p	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Deltamethrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Dichlobenil	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Dicofol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Dieldrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Endosulfan alpha	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Endosulfan beta	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Endrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Esfenvalerat	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Flumioxazin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Fluquiconazol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Fluvalinat	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Folpet	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Heptachlor	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Heptachlorepoxid	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorbenzol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorbutadien	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorhexan alpha	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorhexan beta	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorhexan delta	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Hexachlorhexan gamma	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Iprodion	DIN 38407 F36: 2014-09	µg/l	0.005	<0.005	<0.005	<0.005
Isodrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Kresoxim-methyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Methoxychlor	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Octachlornaphthalin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005

Octachlorstyrol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Parathion-ethyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Parathion-methyl	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Pentachlorbenzol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Pyrethrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Pyridat	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Quinoxyfen	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Quintozen	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Tefluthrin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Tetrachlorbenzol	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Tolylfluanid	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Trichlorbenzol 1.2.3	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Trichlorbenzol 1.2.4	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Trichlorbenzol 1.3.5	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005
Trifluralin	on the basis of DIN EN ISO 6468-F1: 1997-02	µg/l	0.005	<0.005	<0.005	<0.005

n.a. = not available

Appendix table 9: Organotin compounds and pesticides of each tank of the sediment of the benthic and the eulittoral tests during the mesocosom experiment year 1 and 2. The analyses were done according to the mentioned standards.

			habitat	benthic						eulittoral					
			year	1			2			1			2		
			sampling date	09.05.15	09.05.15	09.05.15	05.08.15	05.08.15	05.08.15	08.05.15	08.05.15	08.05.15	05.09.15	05.09.15	05.09.15
Organotin compounds	**standard**	unit	detect-ion limit	tank 1	tank 2	tank 3	tank 1	tank 2	tank 3	tank 1	tank 2	tank 3	tank 1	tank 2	tank 3
Monobutyltin	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Dibutyltin	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Tributyltin	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Tetrabutyltin	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Tricyclohexyltin cation	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Triphenyltin cation	DIN ISO 23161:2011-10	µg/kg	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Pesticides															
Atrazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Acetamiprid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Ametroctadin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Amidosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Azoxystrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Beflubutamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Benalaxyl-M	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Bentazon	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Benthiavalicarb-isopropyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Bixafen	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Boscalid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Bromacil	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Bromoxynil	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Carbendazim	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Carfentrazon-ethyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Chlorfenvinphos	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Chloridazon	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Chlortoluron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Clethodim	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Clodinafop-propagyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Clomazone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Clothianidin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Cyazofamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Cycloxydim	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Cyproconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Cyprodinil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Desethyl-Atrazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Desethylterbutylazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Desisoprpoylatrazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Dicamba	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Dichlorprop	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Difenconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Diflufenican	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Dimethachlor	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Dimethenamid-P	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Dimethoate	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Dimethomorph	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Dimoxystrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Diuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Epoxiconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Ethidimuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Ethofumesat	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Fenamidone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenazaquin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenhexamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenoxaprop-P	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenoxycarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenpropidin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fenpyroxymat	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flazasulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flonicamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Florasulam	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fluazifop-P	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fludioxonil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flufenacet	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fluopicolide	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fluoxastrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flupyrsulfuron-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fluroxypyr-methylhepthyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flurtamone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flusilazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Flutolanil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Foramsulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fosthiazat	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Fuberidazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Glyphosat	on the basis of ISO 21458:2008E	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Haloxyfop-P	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Hexythiazox	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Imazalil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Imazosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2

Imidacloprid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Indoxacarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Ioxynil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Iprovalicarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Isoproturon	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Isoxaben	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Lenacil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Mandipropamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
MCPA	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Mecoprop	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Mepanipyrim	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Mesosulfuron-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Mesotrione	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Metalaxyl-M	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Metamitron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metazachlor	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Methabenzthiazuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Methiocarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Metosulam	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Metoxuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metrafenone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Metribuzin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metsulfuron-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Myclobutanil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Napropamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Nicosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Paclobutrazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Penconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2

Pencycuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pethoxamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Phenmedipham	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Picolinafen	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Picoxystrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pinoxaden	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pirimiphos-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Primicarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Prochloraz	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Propamocarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Propaquizafop	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Propiconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Propoxycarbazone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Propyzamid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Proquinazid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Prosulfocarb	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Prosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Prothioconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pymetrozin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pyraclostrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pyraflufen-ethyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pyrimethanil	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Pyroxsulam	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Quinmerac	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Quinoclamin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Quizalofop-P	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Silthiopham	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Simazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S-Metolachlor	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Sulcotrione	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Sulfosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Tebuconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Tembotrione	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Tepraloxidim	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Terbuthylazin	on the basis of DIN 38407 F36: 2014-09	µg/kg	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Tetraconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thiabendazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thiacloprid	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thiamethoxam	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thiencarbazone-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thifensulfuron-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Thiophanat-methyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Topramezone	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Triasulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Triclopyr-2-buthoxyethyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Trifloxystrobin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Triflusulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Triticonazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Tritosulfuron	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Warfarin	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Zoxamide	on the basis of DIN 38407 F36: 2014-09	µg/kg	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Acequinocyl	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Aclonifen	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Aldrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Bifenox	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Bromophos-ethyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Captan	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorpropham	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorpyrifos-ethyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorpyrifos-methyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorthalonil	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cyfluthrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cyhalothrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cypermethrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDD o.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDD p.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDE o.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDE p.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDT o.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DDT p.p	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Deltamethrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Dichlobenil	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Dicofol	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Dieldrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Endosulfan alpha	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Endosulfan beta	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Endrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Esfenvalerat	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Flumioxazin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fluquiconazol	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fluvalinat	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Folpet	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Heptachlor	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Heptachlorepoxid	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hexachlorbenzol	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Hexachlorbutadien	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hexachlorhexan alpha	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hexachlorhexan beta	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hexachlorhexan delta	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hexachlorhexan gamma	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Iprodion	on the basis of DIN 38407 F36: 2014-09	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Isodrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Kresoxim-methyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Methoxychlor	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Octachlornaphtha-lin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Octachlorstyrol	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Parathion-ethyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Parathion-methyl	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Pentachlorbenzol	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Pyrethrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Pyridat	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Quinoxyfen	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Quintozen	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tefluthrin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tetrachlorbenzol	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tolylfluanid	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Trichlorbenzol 1.2.3	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Trichlorbenzol 1.2.4	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Trichlorbenzol 1.3.5	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Trifluralin	on the basis of DIN ISO 10382:2003-05	µg/kg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Appendix table 10: The disintegrated area (%) of PBSe, PBSeT, PHB and PE samples from the mesocosm benthic test (sublittoral, seafloor scenario), eulittoral (intertidal scenario), and pelagic test (water column scenario) of year 1 and 2. The exposure time of the samples is given in days. MV: Mean value; SD: Standard deviation; n.a.: Not available.

benthic				eulittoral				pelagic			
year 1											
days	polymer	MV	SD	days	polymer	MV	SD	days	polymer	MV	SD
78	PBSe	n.a.	n.a.	79	PBSe	n.a.	n.a.	78	PBSe	n.a.	n.a.
	PBSeT	0.4	0.1		PBSeT	0.4	0.2		PBSeT	0.6	0.1
	PHB	1.2	0.8		PHB	0.6	0.7		PHB	15.8	19.3
	PE	0.0	0.0		PE	0.1	0.1		PE	0.0	0.0
154	PBSe	n.a.	n.a.	153	PBSe	n.a.	n.a.	154	PBSe	n.a.	n.a.
	PBSeT	1.2	0.2		PBSeT	10.3	8.2		PBSeT	2.8	2.6
	PHB	16.1	13.0		PHB	7.7	8.1		PHB	19.2	26.9
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0
229	PBSe	n.a.	n.a.	228	PBSe	n.a.	n.a.	229	PBSe	n.a.	n.a.
	PBSeT	2.2	1.5		PBSeT	32.3	7.0		PBSeT	19.8	26.2
	PHB	32.2	46.8		PHB	10.5	10.1		PHB	5.8	4.6
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0
308	PBSe	n.a.	n.a.	308	PBSe	n.a.	n.a.	308	PBSe	n.a.	n.a.
	PBSeT	8.4	7.4		PBSeT	41.4	6.1		PBSeT	23.8	22.3
	PHB	56.4	36.2		PHB	11.3	13.6		PHB	6.8	2.8
	PE	0.1	0.1		PE	0.0	0.0		PE	0.0	0.1
year 2											
days	polymer	MV	SD	days	polymer	MV	SD	days	polymer	MV	SD
77	PBSe	0.6	0.2	77	PBSe	15.5	13.8	77	PBSe	8.9	9.5
	PBSeT	0.1	0.1		PBSeT	2.4	1.6		PBSeT	0.6	0.5
	PHB	4.0	5.3		PHB	0.9	1.1		PHB	0.2	0.2
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0
151	PBSe	n.a.	n.a.	152	PBSe	n.a.	n.a.	151	PBSe	n.a.	n.a.
	PBSeT	0.8	0.3		PBSeT	75.1	34.8		PBSeT	14.8	4.2
	PHB	27.9	19.7		PHB	11.8	0.3		PHB	8.2	5.8
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0
237	PBSe	1.3	0.5	238	PBSe	90.2	7.5	237	PBSe	2.6	1.4
	PBSeT	11.6	10.5		PBSeT	91.7	6.2		PBSeT	39.7	21.7
	PHB	28.5	12.8		PHB	37.4	9.5		PHB	4.3	6.6
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0
271	PBSe	n.a.	n.a.	270	PBSe	n.a.	n.a.	271	PBSe	n.a.	n.a.
	PBSeT	13.2	5.2		PBSeT	57.4	4.2		PBSeT	86.9	14.0
	PHB	24.7	10.2		PHB	8.7	3.2		PHB	10.9	11.2
	PE	0.0	0.0		PE	0.0	0.0		PE	0.0	0.0

Appendix table 11: Structures proposed for PBSe when measured with MALDI-TOF.

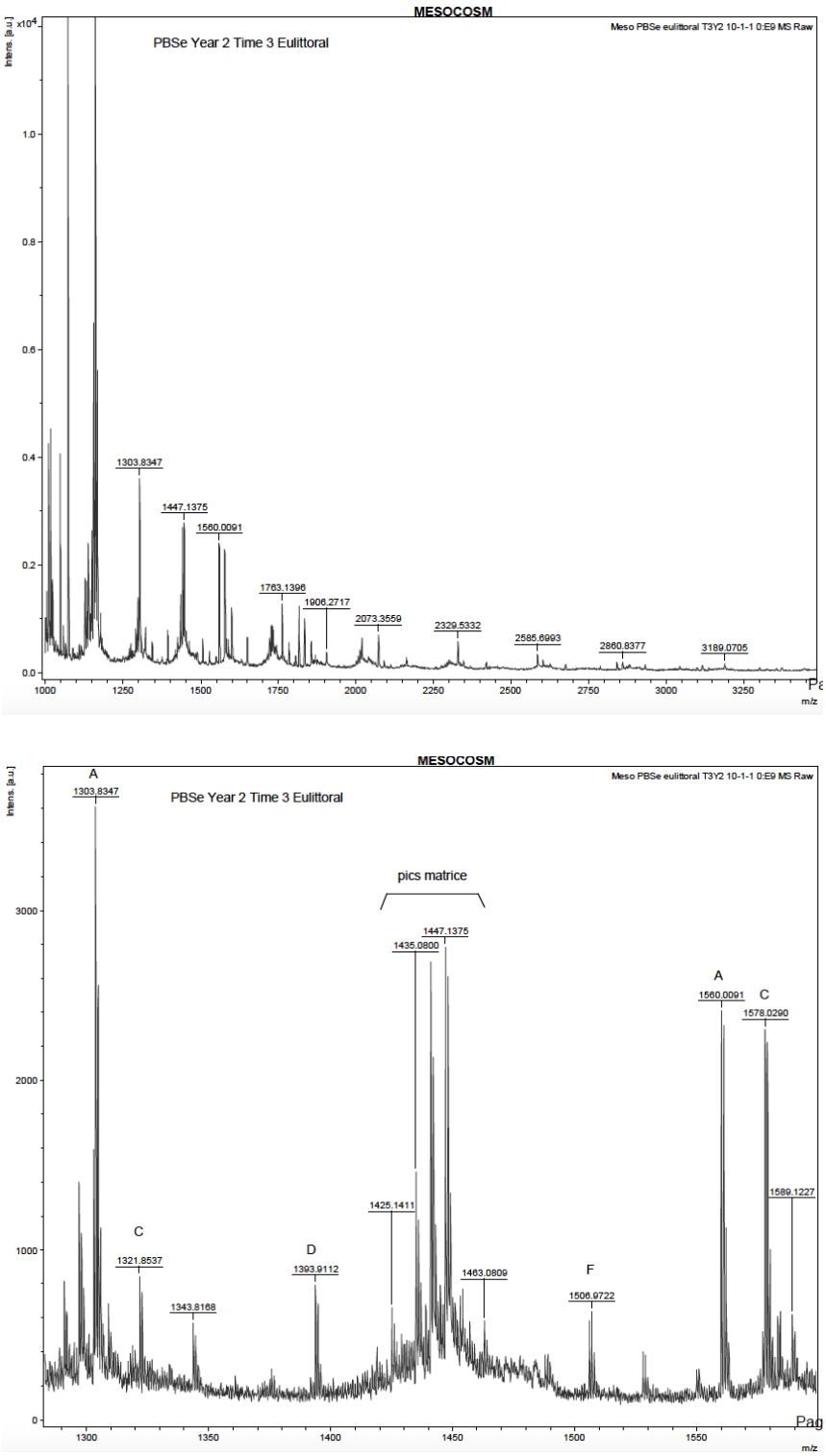
Distribution	Mass of end chains observed (Da)	Proposed structures
A	0	Cyclic structure $-(C_{14}H_{24}O_4)_n-$ + Na^+ with n= 4 to n= 14
B	0	Cyclic structure $-(C_{14}H_{24}O_4)_n-$ + K^+ with n= 4 to n= 14
C	18	$H-(C_{14}H_{24}O_4)_n-OH$ + Na^+ with n= 4 to n= 14
D	90	$H-(C_{14}H_{24}O_4)_n-O-(CH_2)_4-OH$ + Na^+ with n= 4 to n= 14
E	184	Not defined
F	202	Not defined

Appendix table 12: Detected ions of PBSeT with a cyclic distribution listed in bold, measured with MALDI-TOF.

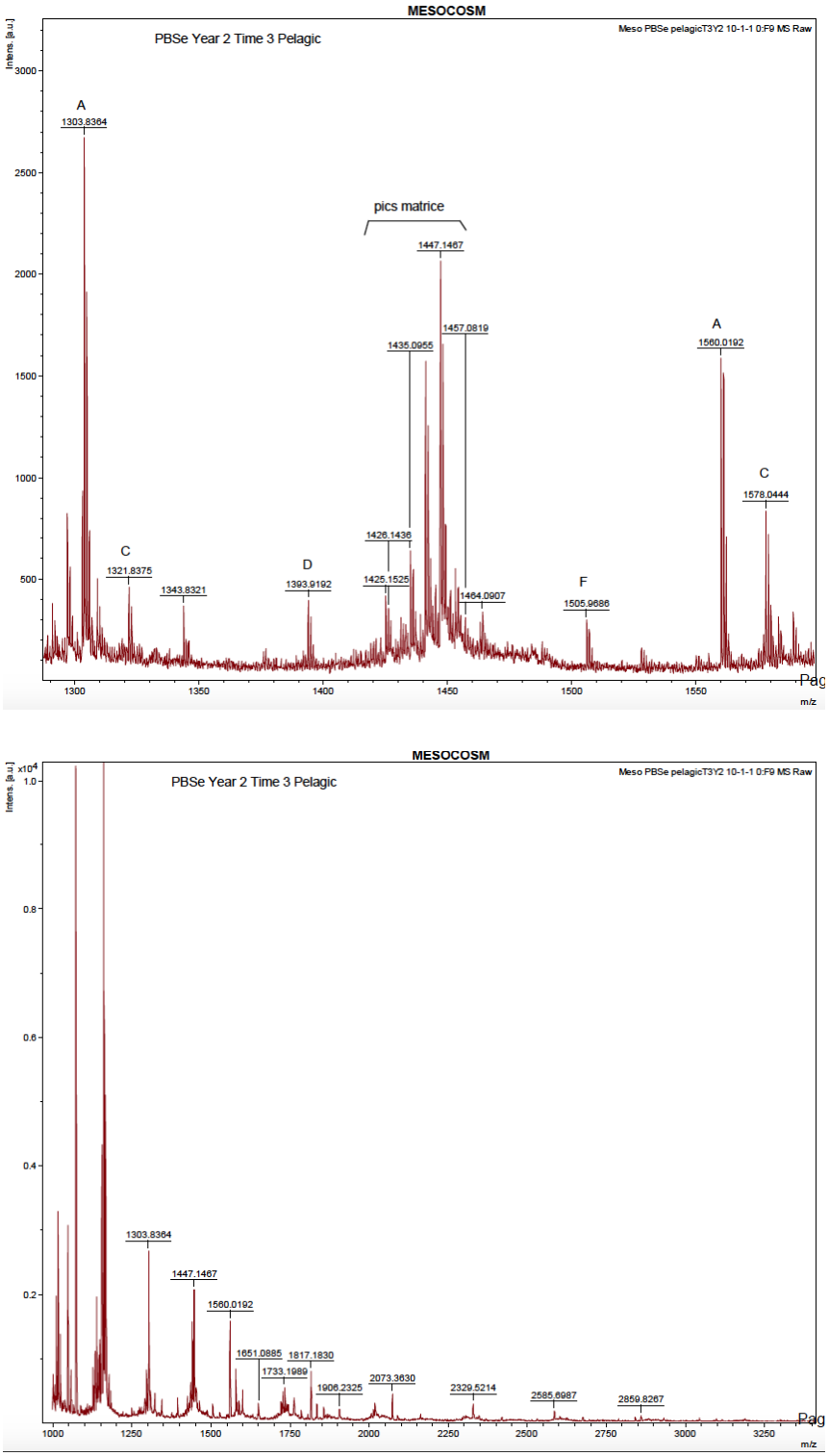
n	m						
	0	1	2	3	4	5	6
0	22,9892	243,0622	463,1352	683,2082	903,2812	1123,3543	1343,4273
1	279,1561	499,2291	**719,3021**	**939,3751**	1159,4482	1379,5212	1599,5942
2	535,3230	**755,3960**	**975,4690**	**1195,5421**	**1415,6151**	**1635,6881**	**1855,7611**
3	**791,4899**	**1011,5629**	**1231,6360**	**1451,7090**	**1671,7820**	**1891,8550**	**2111,9280**
4	**1047,6568**	**1267,7299**	**1487,8029**	**1707,8759**	**1927,9489**	**2148,0219**	2368,0949
5	1303,8238	**1523,8968**	**1743,9698**	**1964,0428**	**2184,1158**	**2404,1888**	2624,2618
6	1559,9907	1780,0637	**2000,1367**	**2220,2097**	**2440,2827**	**2660,3557**	2880,4287
7	1816,1576	2036,2306	2256,3036	2476,3766	2696,4496	2916,5226	3136,5956

Appendix table 13: Detected ions of PBSeT with a linear distribution listed in bold, measured with MALDI-TOF.

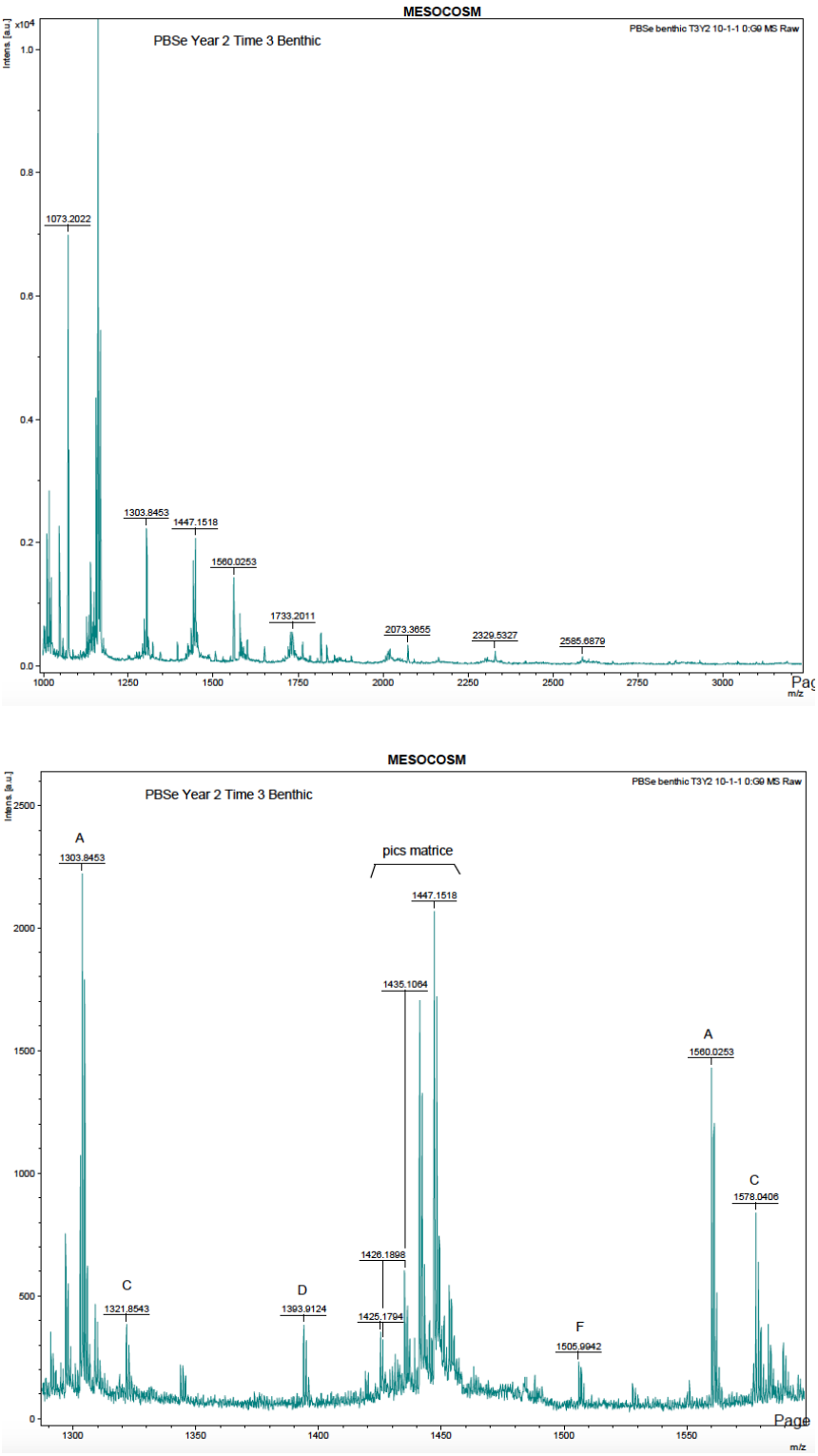
b	a						
	0	1	2	3	4	5	6
0	40.9992	261.0722	481.1452	701.2182	921.2912	1141.3643	1361.4373
1	297.1661	517.2391	737.3121	957.3851	**1177.4582**	**1397.5312**	**1617.6042**
2	553.3330	773.4060	993.4790	**1213.5521**	**1433.6251**	1653.6981	1873.7711
3	809.4999	**1029.5729**	**1249.6460**	**1469.7190**	**1689.7920**	1909.8650	2129.9380
4	1065.6668	**1285.7399**	**1505.8129**	1725.8859	1945.9589	2166.0319	2386.1049
5	1321.8338	1541.9068	1761.9798	1982.0528	2202.1258	2422.1988	2642.2718
6	1578.0007	1798.0737	2018.1467	2238.2197	2458.2927	2678.3657	2898.4387
7	1834.1676	2054.2406	2274.3136	2494.3866	2714.4596	2934.5326	3154.6056



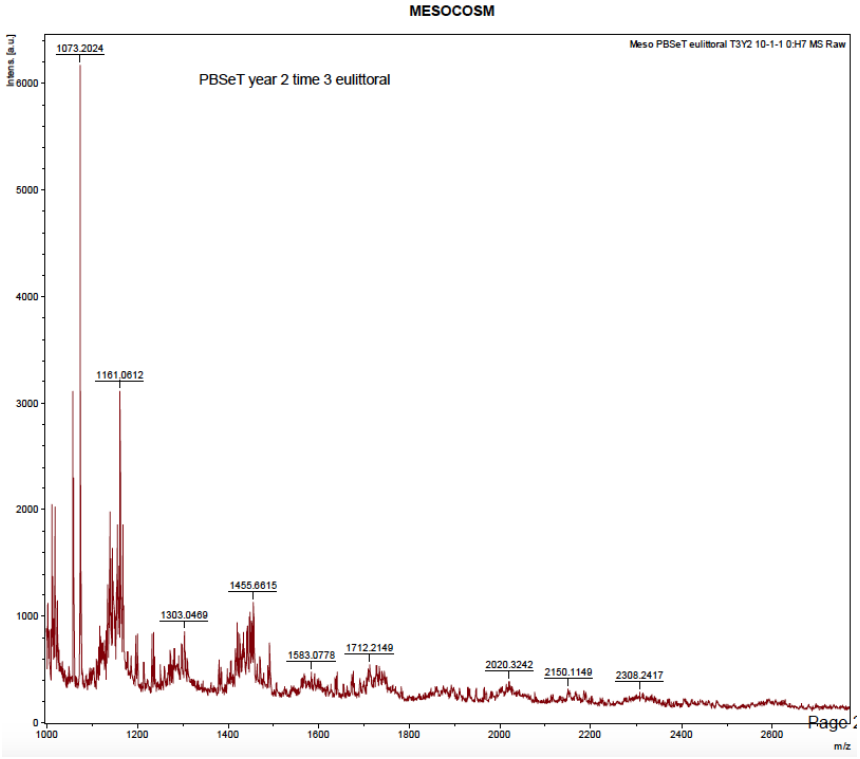
Appendix figure 1: MALDI-TOF Mass spectra of a PBSe samples exposed in the eulittoral test (intertidal, beach scenario) for 238 days.

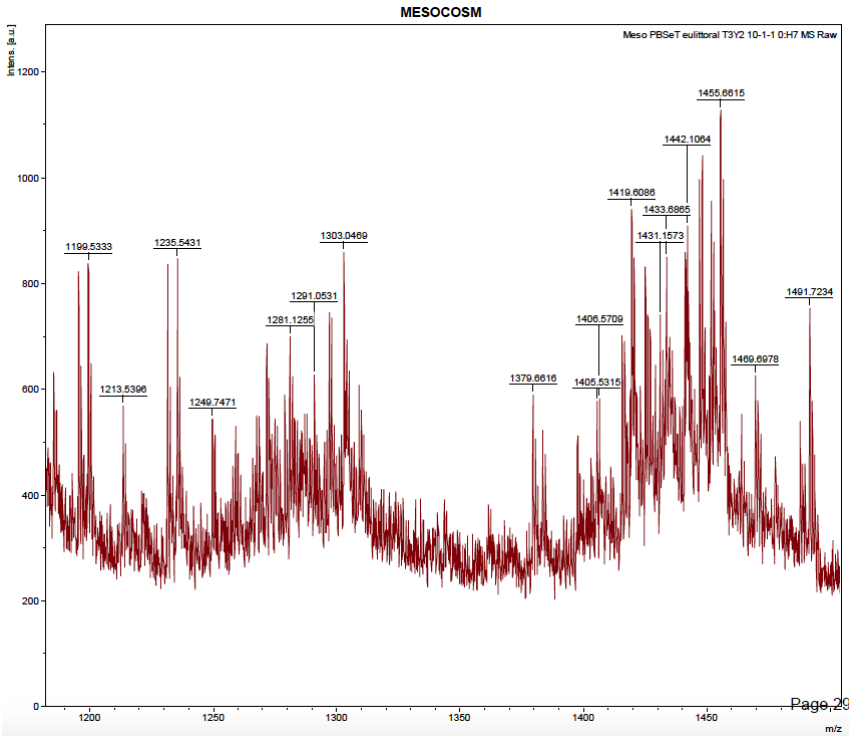


Appendix figure 2: MALDI-TOF Mass spectra of a PBSe samples exposed in the pelagic test (water column scenario) for 238 days.

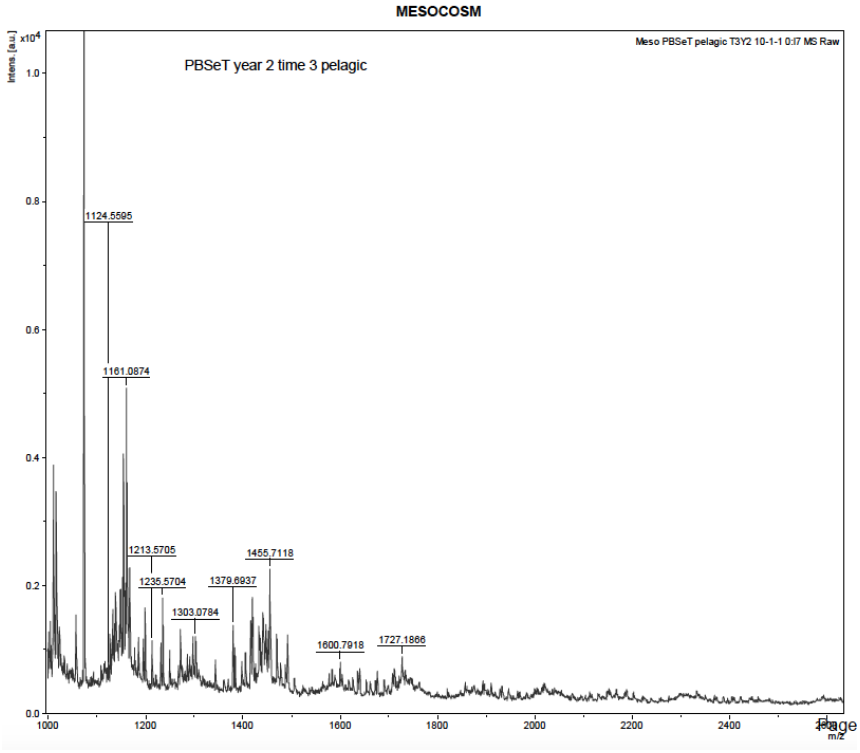


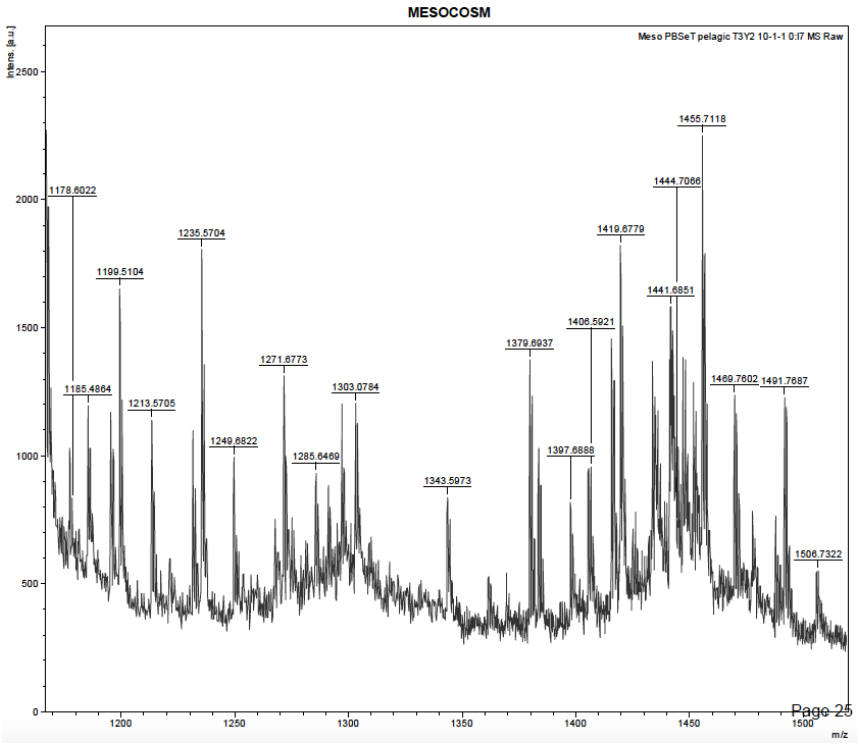
Appendix figure 3: MALDI-TOF Mass spectra of a PBSe samples exposed in the benthic test (sublittoral, seafloor scenario) for 238 days.



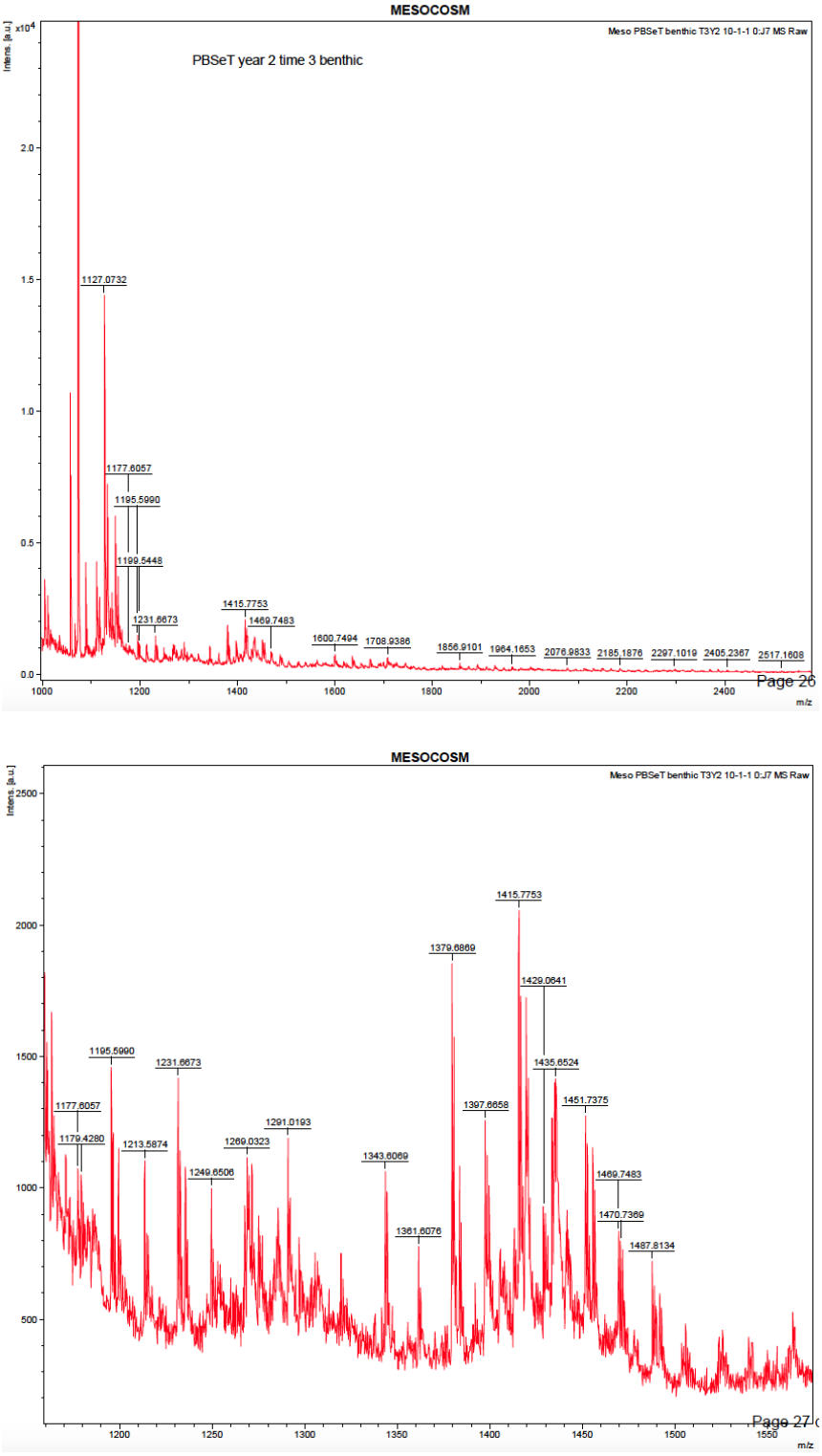


Appendix figure 4: MALDI-TOF Mass spectra of a PBSeT samples exposed in the eulittoral test (intertidal, beach zone scenario) for 238 days.





Appendix figure 5: MALDI-TOF Mass spectra of a PBSeT samples exposed in the pelagic test (water column scenario) for 238 days.



Appendix figure 6: MALDI-TOF Mass spectra of a PBSeT samples exposed in the benthic test (sublittoral, seafloor scenario) for 238 days.

Appendix table 14: The tensile properties of PBSeT and PHB samples from the mesocosm eulittoral (intertidal, beach scenario), pelagic (water column scenario) and benthic test (sublittoral, seafloor scenario) from year 1 (a) and 2 (b). PBSe was only exposed in year 2 and sampled at two intervals. tq is the untreated original polymer. Measured were strength at break (SaB), elongation at break (Elong), and thickness (Th). Displayed is the mean value and its standard deviation of three replicates. t_1 – t_4 are the sampling intervals of ca. 2.5 months each. The exact sampling dates are listed in table 2. n.d.: not determinable; n.a.: not available

Year 1												
Table a)	t_1			t_2			t_3			t_3		
PBSe	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)
tq	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**
benthic	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
eulittoral	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
pelagic	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PBSeT												
tq	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**
benthic	2.9 ± 0.1	16.3 ± 7.3	0.025 ± 0.001	n.d.	n.d.	n.d.	7.2	19.4	0.026	n.d.	n.d.	n.d.
eulittoral	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pelagic	4.9	21.9	0.031	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PHB												
tq	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**
benthic	7.7 ± 3.0	18.3 ± 12.0	0.065 ± 0.009	n.d.	n.d.	n.d.	9.2 ± 1.09	19.1 ± 5.10	0.070 ± 0.009	8.5 ± 3.1	15.1 ± 12.5	0.074 ± 0.003
eulittoral	12.1 ± 2.8	33.8 ± 19.3	0.082 ± 0.008	8.6 ± 1.5	13.1 ± 4.7	0.079 ± 0.002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pelagic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
LDPE												
tq	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**
benthic	29.8 ± 1.7	130.9 ± 24.0	0.029 ± 0.002	31.3 ± 2.7	155.1 ± 24.8	0.028 ± 0.001	30.8 ± 1.59	137.5 ± 7.82	0.029 ± 0.002	30.4 ± 1.5	138.1 ± 24.3	0.030 ± 0.002
eulittoral	31.0 ± 1.5	141.9 ± 13.2	0.030 ± 0.001	32.5 ±1.7	170.2 ± 9.7	0.029 ± 0.002	31.1 ± 2.98	124.8 ± 25.23	0.026 ± 0.002	29.9 ± 1.0	130.2 ± 11.8	0.030 ± 0.002
pelagic	31.2 ± 1.2	157.9 ± 19.7	0.029 ± 0.002	31.3 ± 1.7	156.1 ± 10.2	0.028 ± 0.001	29.4 ± 0.97	133.7 ± 11.09	0.030 ± 0.03	30.8 ± 1.5	133.0 ± 23.3	0.028 ± 0.002

Year 2												
Table b)	t_1			t_2			t_3			t_3		
PBSe	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)	SaB (Mpa)	Elong (%)	Th (mm)
tq	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**	**36.4 ± 2.6**	**698.6 ± 74.6**	**0.026 ± 0.001**
benthic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
eulittoral	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pelagic	6.7 ± 2.3	1.6 ± 0.4	0.023 ± 0.001	6.2±1.4	1.2±0.3	0.024±0.003	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBSeT												
tq	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**	**39.1 ± 3.0**	**412.9 ± 31.2**	**0.024 ± 0.002**
benthic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
eulittoral	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pelagic	1.5	7	0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PHB												
tq	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**	**22.9 ± 0.9**	**232.0 ± 20.9**	**0.092 ± 0.003**
benthic	4.7 ± 0.5	7.2 ±4.2	0.056 ± 0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
eulittoral	10.5 ± 2.2	20.5 ± 3.1	0.070 ± 0.009	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pelagic	9.2 ± 4.3	18.3 ± 14.6	0.063 ± 0.014	10.0±2.1	19.8±9.1	0.055±0.01	9.4±2.2	13.0±3.8	0.061±0.015	n.d.	n.d.	n.d.
LDPE												
tq	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**	**31.8 ± 1.4**	**189.7 ± 30.0**	**0.028 ± 0.002**
benthic	31.5 ± 1.9	136.3 ± 29.8	0.027 ± 0.002	28.8±2.0	117.6±23.9	0.030±0.001	27.9±1.7	126.6±25.2	0.030±0.001	29.2±1.9	134.4±27.8	0.029±0.001
eulittoral	31.3 ± 1.7	120.9 ± 19.1	0.029 ± 0.001	30.1±2.8	125.4±20.8	0.029±0.001	30.1±2.7	137.2±13.1	0.031±0.001	32.0±2.8	148.9±31.9	0.027±0.001
pelagic	30.4 ± 2.8	131.8 ± 25.5	0.029 ± 0.001	27.6±1.8	128.4±21.9	0.032±0.002	25.6±3.4	112.0±28.1	0.031±0.002	27.5±1.8	116.1±27.3	0.029±0.001