Le ali alle tue idee

Eco-sustainable production of polyhydroxyalkanoates (PHAs) and their uses in wide range of applications

Speakers:

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Prof. Maurizia Seggiani – *Dept. of Civil and Industrial Engineering, University of Pisa, Pisa, Italy*

Project Team

- **Femto Engineering srl**
- **Zefiro srl**
- **Cooperativa Sociale Giovanile di Lavoro** – C.S.G.L.
  - **Department of Civil and Industrial Engineering** - University of Pisa
  - **Department of Biology** - University of Pisa
  - **Istitute of Ecosystem Study –** National Research Council
    - **Laboratori ARCHA srl**

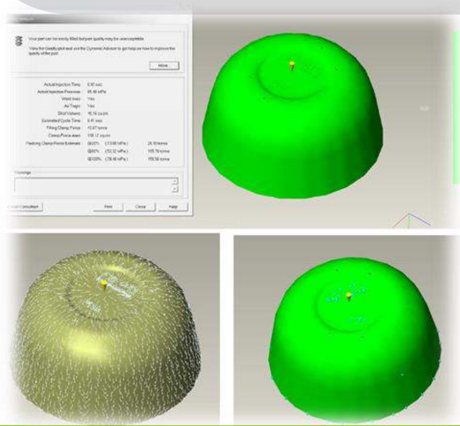
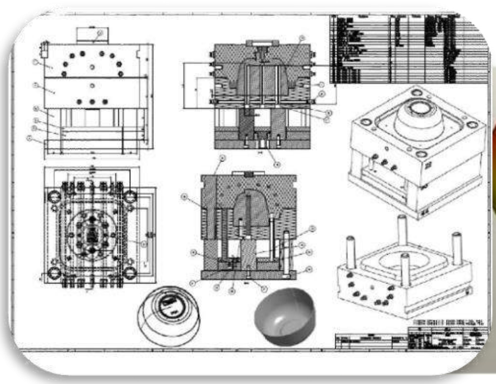
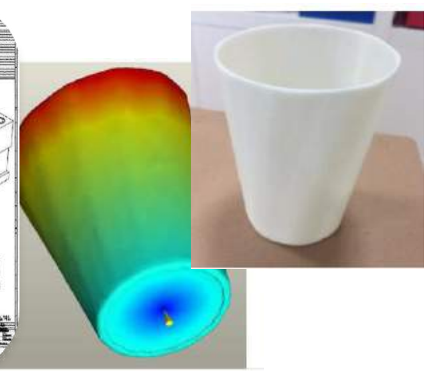
PHA Project Goals

- Development and optimization of a **new low-cost production of PHAs** based on specific **photosynthetic bacteria** in new generation photo-bioreactors using agro-food industrial wastewaters

- Development of **PHA-based bio-composites** and **natural fibers** such as lignocellulosic waste fibers such as **saw dust** and *Posidonia oceanica* residues deposited on the beaches (*egagropili*) for use in terrestrial and dune/marine environments, respectively.

PHA Project Goals

- **Design and Production by injection moulding** of the following **demonstrators**:
  - pots, grids and plant tutors using the optimized composite based on PHB and fibres of *P. oceanica* for applications in dune/marine environments (restoration of marine habitats).
  - pots using the optimized composites based on PHA and saw dust for applications in plant nursery;
  - small food-contact containers using the composites based on PHA and bran fibres.

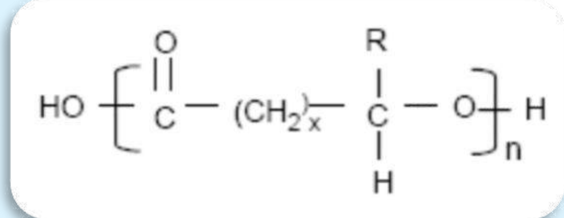

PHA Project Goals

- **Validation of the demostrators**

  Degradability tests in dune/marine mesocosmos for the composites based on PHA and ***egagropili***

  Phytotoxicity and composting tests for composites based on PHA and **saw dust**.

What are PHAs?

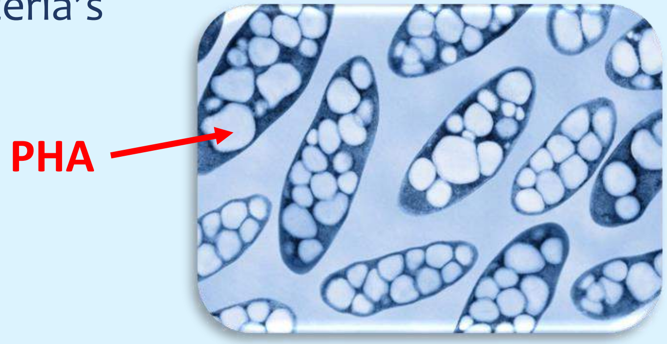


Polyhydroxyalkanoates (PHAs) are **biological thermoplastic polyesters** produced by numerous microorganisms through bacterial fermentation of sugar or lipids.

They are intracellular carbon and energy storage compounds, produced under **limiting nutritional conditions** of N, P, S, Mg, etc. and in presence of excess of carbon.
Many companies produce PHAs using pure culture of *Ralstonia eutropha*.

The PHA granules can represent up to 90% of the bacteria's dry weight. PHAs can produced by many bacteria (Bacillus, Rhodococcus, Pseudomonas, etc...).

Escherichia coli
with PHA granules



Why PHA?

PHA have **good thermal stability** (melting points 160-180°C) e **can be processed as the conventional termoplastics.**

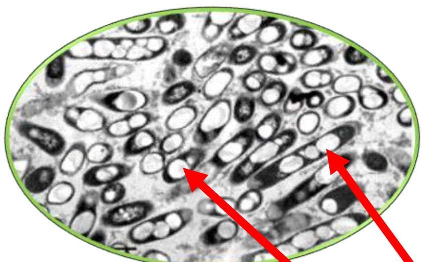
PHAs are among the few polymers **biodegradable** both in soil and in **sea water**

A number of bacteria are producers of PHA

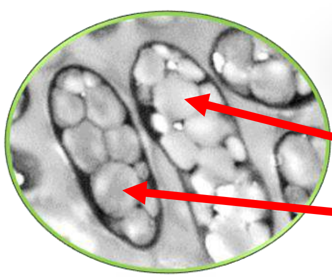
Two different processes are shown on this slide for PHA production

Fermentation

Carried out by heterotrophic bacteria



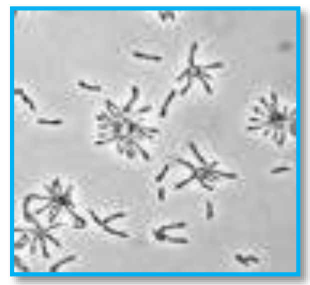
Pseudomonas sp.



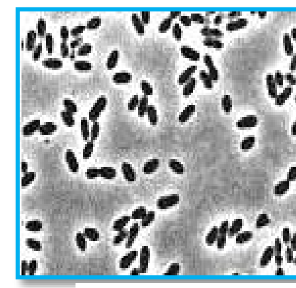
Ralstonia eutropha

Photofermentation

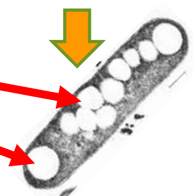
Carried out by purple bacteria



Rhodopseudomonas palustris

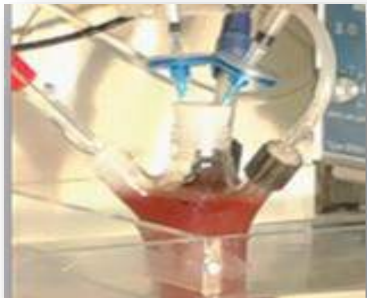


Rhodobacter sphaeroides



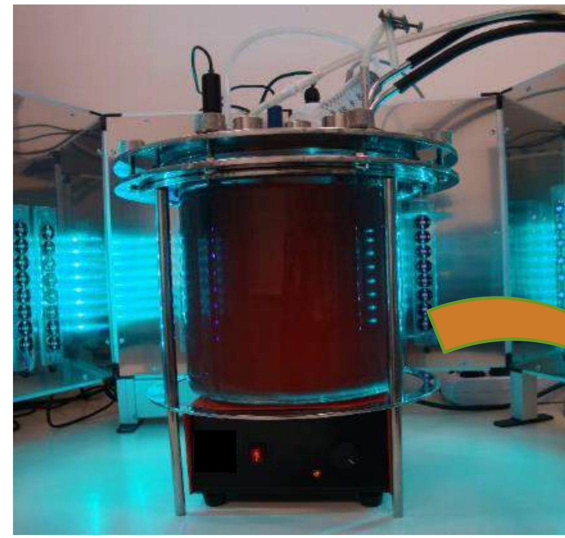
PHA Granules into bacterial cells

Lab-scale photobioreactors with working volumes ranging from 0.25 to 4.0 L

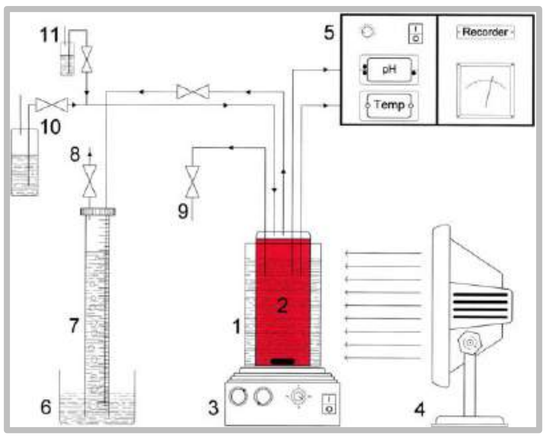


Working volume = 0.25 L

Working volume = 1.0 L



Working volume = 4.0 L



*Schematic diagram of cultural system for combined production of H_2 and **PHB**

PHB

PHB-rich biomass

*Carlozzi P. (2012). J Biomed Biotechnol 8. Article ID 590693, doi: 10.1155/2012/590693

Results obtained by feeding *Rhodopseudomonas* sp.1 with pure carbon sources

Lab-scale PBR
Vol. = 0.25 L

TAB. 1 *Rhodopseudomonas* sp. 1 fed with pure carbon sources

Modified van-Niel's medium		Cell concentration (g/L)		PHB content	
Carbon source	**Nutrient deficiency**	**Start**	**End**	**(mg/L)**	**(%)**
Acetic acid	Nitrogen	0.22	1.82	93	5.1
Butyric acid†		0.24	2.32	156	6.7
Butyric acid*		0.24	2.57	192	7.5
Butyric acid	Nitrogen	3.10	5.24	727	13.9
Butyric acid†	Phosphate	1.80	1.95	178	9.2
Lactic acid†		0.21	2.24	14	0.6
Lactic acid*		0.16	4.22	268	6.4

(†) NH_4Cl; (*) Glutamate

The highest PHB content was obtained with butyric acid under nitrogen deficiency

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Indoor PHB-production by purple bacteria fed with dairy effluents

Dairy wastewater → Fermentation → Fermented dairy

TAB. 2. Indoor growth of *Rhodopseudomonas* sp. (strains 1 e 4) fed with different pretreated dairy effluents* diluted with water.

Rhodopseudomonas strains	Culture broths	VFAs (g/L)		COD (mg/L)		Biomass productivity	PHB (%)
		Start	End	Start	End	mg/L d	
1	FD 25%	5.3	2.2	8042	4317	86	18
1	FD 50%	10.6	4.6	16084	9233	74	10
4	FD 30%	8.8	2.8	10140	3910	149	52

(*) Effluents obtained by fermentation; (FD) fermented dairy; (VFAs) volatile fatty acids; (COD) chemical oxygen demand

Lab-PBR (4 L)

Biomass

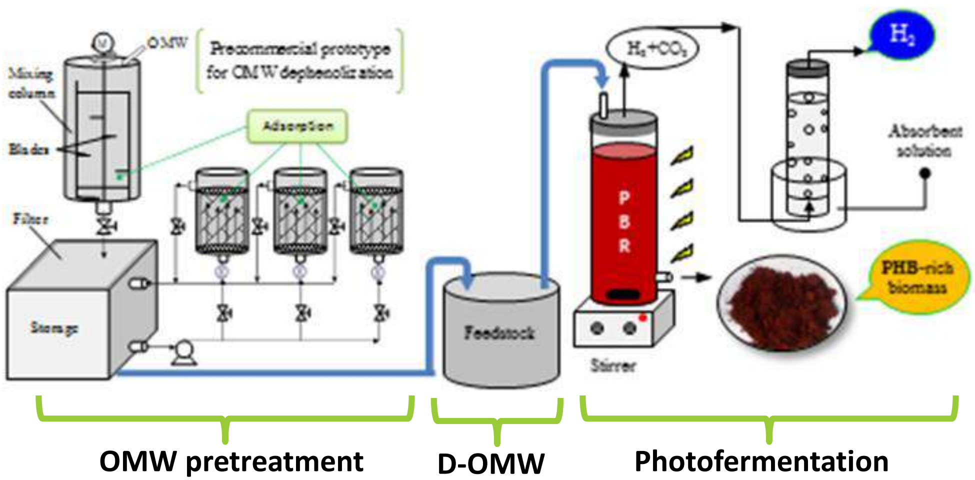
The highest PHB content of 52% of dry-biomass was achieved with the strain 4 using 30% of **fermented dairy** diluted with water (v:v)

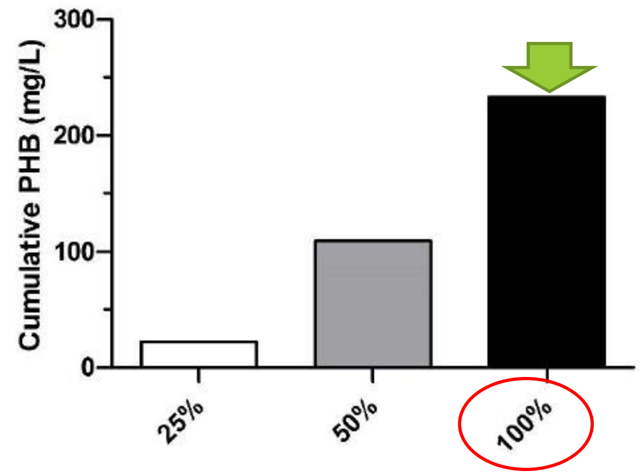
Exausted water broth

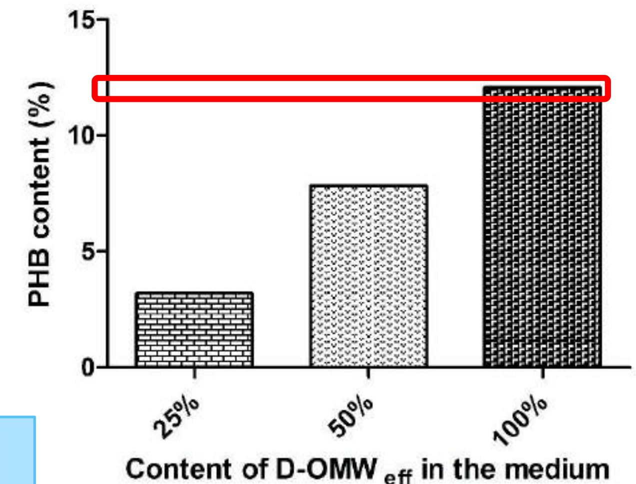
Depigmentation → PHB extraction →

PHB-production by *Rhodopseudomonas* sp. 1 fed with dephenolised OMW

Schematic process







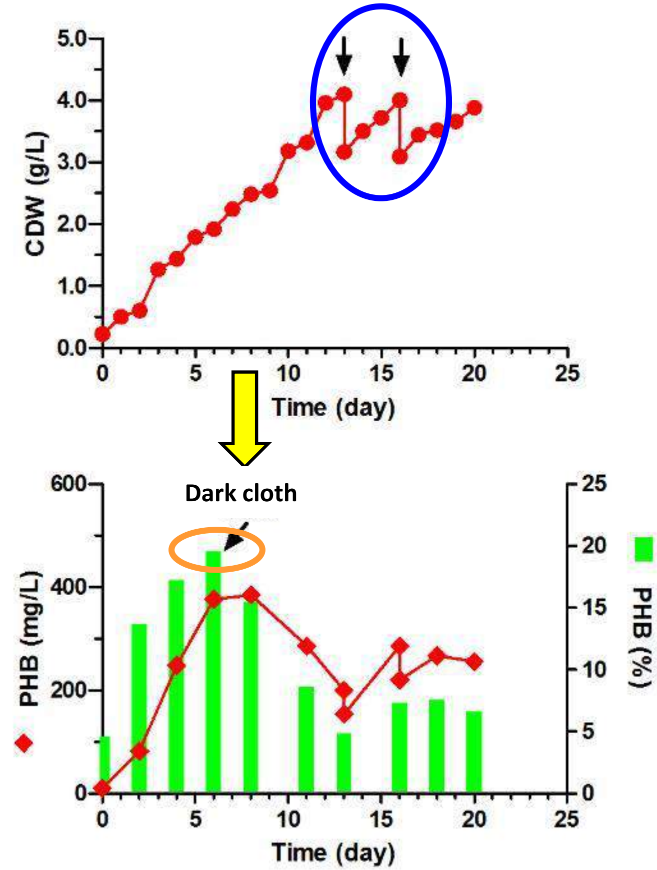
The highest the effluent (D-OMW) content in the culture broth the highest the cumulative PHB

The maximum PHB content in the dry biomass was about 12%

A combined production of bioH_2 was obtained when feeding bacteria with D-OMW (data not shown)

Outdoor PHB production by *Rhodopseudomonas* sp. 1

PBR with 70 L working volume



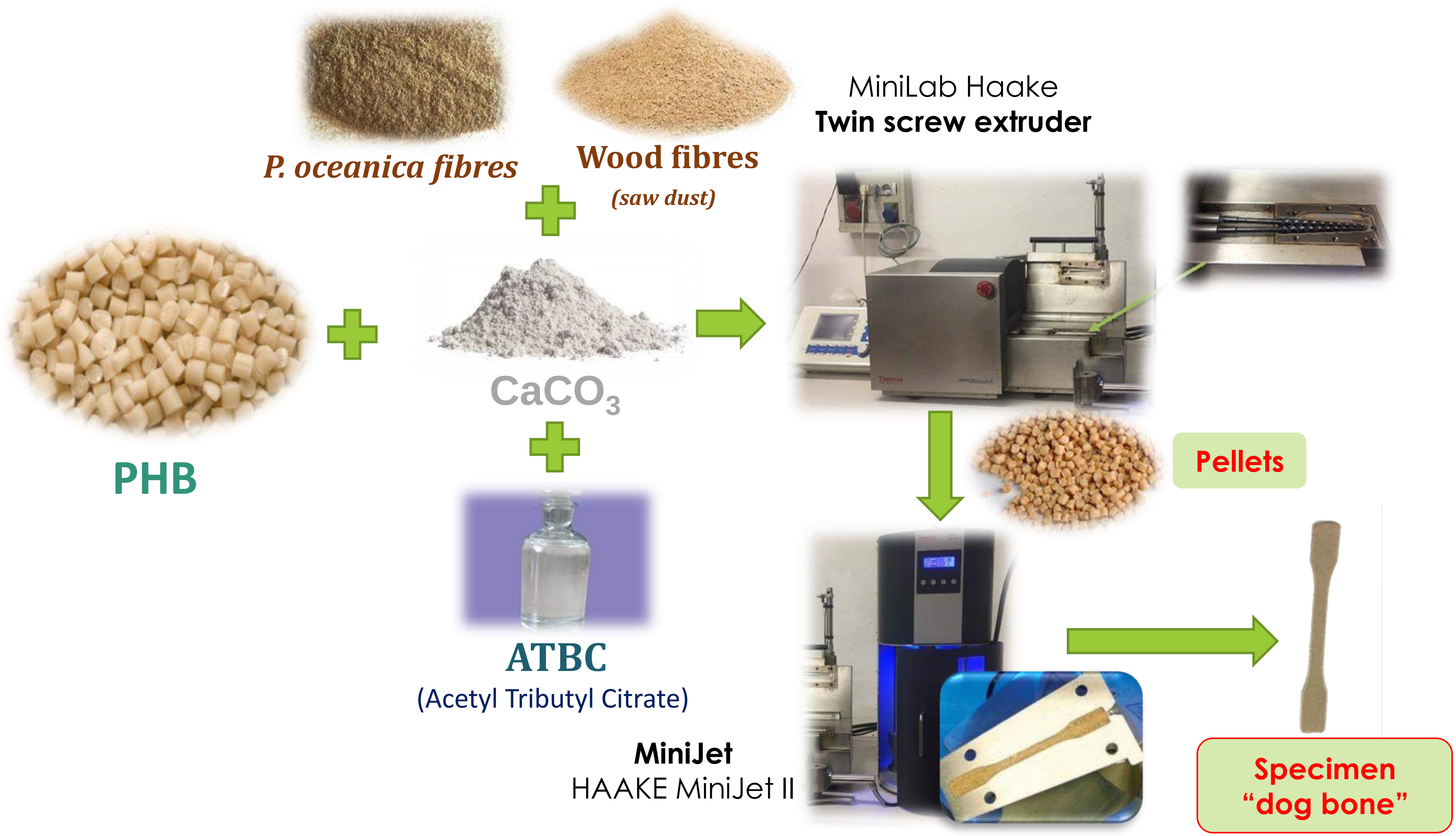
Over 12 days CDW reached a concentration of 4 g/L; the next period the culture was diluted two times

The amount of PHB increased quickly reaching a content of about 20% of CDW, after 6-days

The amount of PHB dropped after covering the reactor to avoid temperature rising


PHB

Production of the composites based on PHB and natural fibres on lab scale



Egagropili: collection and pre-treatments

Egagropili COLLECTION

Drying outdoors

SAND REMOVING
by rotating-
vibrating sieve

WASHING
with fresh water

Sand-free/washed egagropili

DRYING
at 50°C

GRINDING equipped
with vibrating sieve

***P. oceanica* fibres**
(L = 1-2 mm)

Selection of the best formulations

Processability → **Rheological tests and injection moulding**

Torque evaluation, a measurement related to the melt viscosity

dog-bone specimens

before

after break

Mechanical performance → **Tensile tests**

Evaluation of **Young's modulus**, **elongation** and **stress at break**

Instron 5500 R

Impact resistance → **Charpy impact test**

Evaluation of the **amount of energy absorbed** by material during fracture.

Charpy Pendulum

V-notched specimens

before

after break

Selection of the best formulations based on *P. oceanica* fibres

Torque (N•m) at 170°C

Formulation	Time (s)						
	0	10	20	30	40	50	60
Serie PHA/$CaCO_3$ = 95/5							
+ 10 wt.% ATBC + 0 wt. % egagropili	66.3	57. 7	56. 3	55.3	54. 3	54.0	**53**
+10 wt. % ATBC + 10 wt.% egagropili	91	70	68.7	67.3	67.7	67.0	**66**
+10 wt. % ATBC + 10 wt.% egagropili	85	72. 7	71.3	71	69. 7	68. 7	**67**
Serie PHA/$CaCO_3$/ATBC = 85/5/10							
0 wt. % egagropili	64	55.7	55	54.3	54.3	53. 7	**54**
+ 10 wt.% egagropili	87.5	65	64	63.5	63.5	62.0	**61**
+ 20 wt.% egagropili	84.7	62.7	61.7	61	61.3	59.3	**58.6**
+ 30 wt.% egagropili	93.3	85.3	82	80. 7	79. 3	76. 7	**76.6**

Tensile test

Formulation	E(GPa)	ε_r (%)	σ_r (MPa)
Serie PHA/$CaCO_3$ = 95/5			
+ 10 %ATBC +10% egagropili	2.37	2.63	22.23
+ 10%ATBC + 20% egagropili	2.72	2.45	24.62
Serie PHA/$CaCO_3$/ATBC = 85/5/10			
+ 10 % egagropili	1.62	3.26	23.62
+ 20 % egagropili	1.89	2.63	21.52
+ 30 % egagropili	2.27	2.02	22.35

Impact test

Formulation	kJ/m^2
Serie PHA/$CaCO_3$ = 95/5	
+ 10 wt.% ATBC	3.61
+ 10 wt.% ATBC + 10 wt.% egagropili	3.83
+ 10 wt.% ATBC + 20 wt. % egagropili	4.14
+ 10 wt.% ATBC + 30 wt. % egagropili	4.37
Serie PHA/$CaCO_3$/ATBC = 85/5/10	
+ 10 wt.% ATBC	3.61
+ 9 wt.% ATBC + 10 wt.% egagropili	3.67
+ 8 wt.% ATBC + 20 wt.% egagropili	3.93
+ 7 wt.% ATBC + 30 wt.% egagropili	3.97

up to 20 wt. % of *P. oceanica* fibres

- ✓ No moulding problem
- ✓ Good mechanical properties
- ✓ Good impact resistance

Selection of the best formulations based on wood fibres (sawdust)

Torque (N•m) at 170°C

Formulation	Time (s) 0	20	40	60
80%PHA+10%$CaCO_3$+10% plast	**77**	**63.7**	**61.7**	**60.7**
75%PHA+15%$CaCO_3$+10% plast	69.7	59.3	57.7	56
70%PHA+20%$CaCO_3$+10% plast	59.3	53.7	53.3	52
72%PHA+9%$CaCO_3$+9%plast+10 % sawdust	**67.7**	**62**	**60**	**59.3**
68%PHA+8.5%$CaCO_3$+8.5%plast+15 % sawdust	**69.3**	**64.7**	**64**	**61**
64% PHA +8%$CaCO_3$+8%plast+20 % sawdust	85	76.7	75.3	72.0
67.5%PHA+13.5%$CaCO_3$+9% plast+10% sawdust	77.3	70	69	66
63.75% PHA + 12.75% $CaCO_3$+ 8.5%plast +15% sawdust	77	72.3	69.3	67.3
60 % PHA +15% $CaCO_3$+8%plast +20% sawdust	82.7	72.7	71.3	68.7
63 % PHA +18% $CaCO_3$+ 9%plast +10% sawdust	76.3	69.9	67.7	65.7
59.5%PHA+20% $CaCO_3$+8.5%plast +15% sawdust	**72.7**	**67**	**65.7**	**63**
56% PHA +16% $CaCO_3$+8%plast +20% sawdust	84	77.3	73.3	72.3

Tensile Test

Formulation	Modulus (GPa)	ε_r (%)	σ_r (MPa)
80%PHA+10%$CaCO_3$+10% plast	2.64	2.14	25.62
75%PHA+15%$CaCO_3$+10% plast	2.63	2.5	24.9
70%PHA+20%$CaCO_3$+10% plast	2.49	2.66	23.93
72%PHA+9%$CaCO_3$+9% plast+10 % sawdust	**2.35**	**2.05**	**21.02**
68%PHA+8.5%$CaCO_3$+8.5%plast+15 % sawdust	**2.52**	**1.35**	**18.52**
64% PHA +8%$CaCO_3$+8% plast+20 % sawdust	2.94	1.35	20.93
67.5%PHA+13.5%$CaCO_3$+9% plast+10% sawdust	2.8	1.77	20.91
63.75% PHA + 12.75% $CaCO_3$+ 8.5% plast +15% sawdust	3.05	1.34	20.10
60 % PHA +15% $CaCO_3$+8% plast +20% sawdust	3.07	1.25	19.67
63 % PHA +18% $CaCO_3$+ 9% plast +10% sawdust	2.48	1.59	18.68
59.5% PHA +20% $CaCO_3$+8.5% plast +15% sawdust	**2.47**	**1.69**	**18.38**
56% PHA +16% $CaCO_3$+8% plast +20% sawdust	2.91	1.20	18.85

Impact test

Formulation	kJ/m^2
80% PHA + 10% $CaCO_3$ + 10% ATBC	3.57
72% PHA + 9% $CaCO_3$ + 9% ATBC + 10 % sawdust	6.17
68% PHA + 8.5% $CaCO_3$ + 8.5% ATBC + 15 % sawdust	12.24
59.5% PHA + 20% $CaCO_3$+ 8.5% ATBC +15% sawdust	6.2

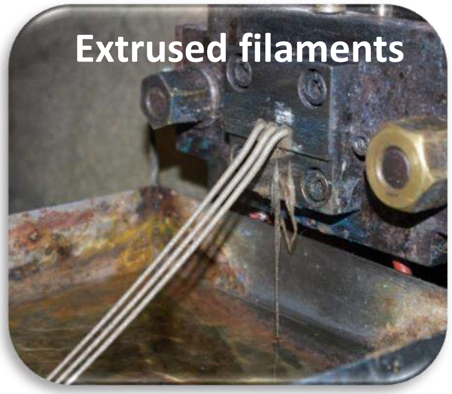
up to 20 wt. % of sawdust
- ✓No moulding problem
- ✓Good tensile properties
- ✓Very good impact resistance

Industrial compounding

Pre-mixing of the compounds

Turbomix

Processing
by twin screw extruder



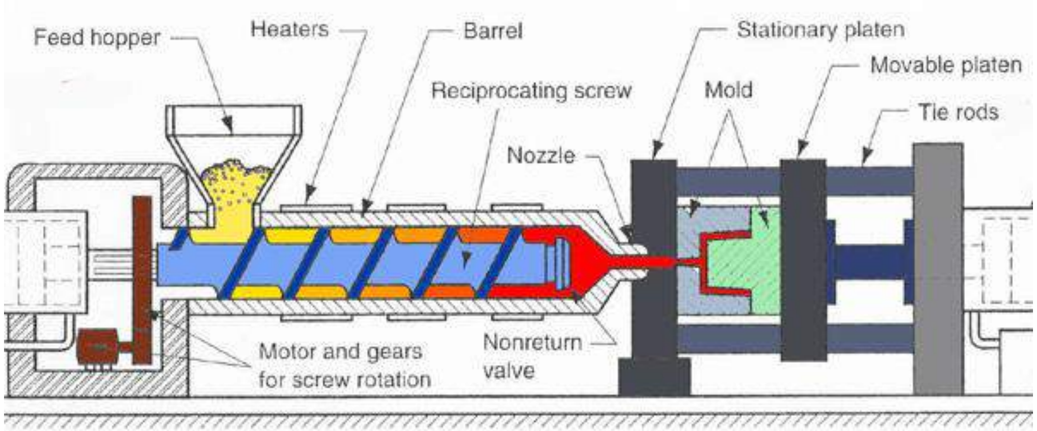
COOLING IN
WATER BATH

Drying

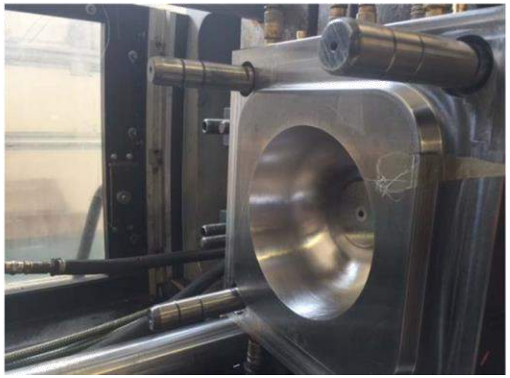
**Dried pellets
for the injection moulding**

INJECTION MOULDING OF THE DEMONSTRATORS

Injection moulding machine

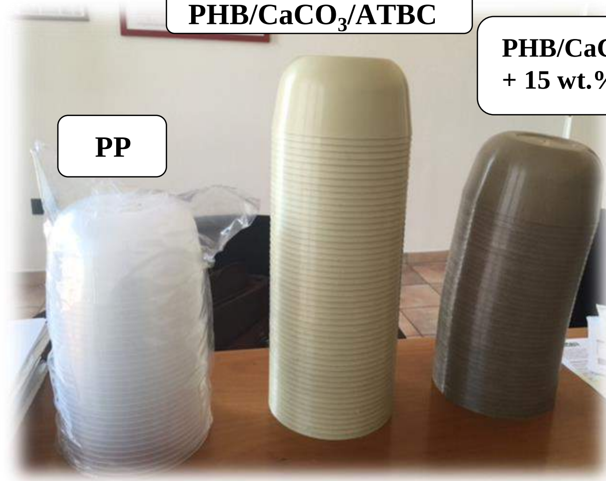


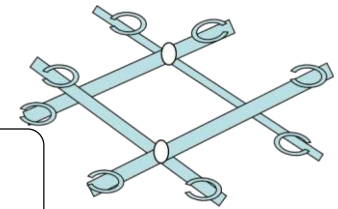
Injection moulding machine «Sandretto» model HP120



Fixed part and mobile part of the mould of the "marine" pot

POTS, GRIDS AND TUTORS FOR MARINE PLANTS based on PHB and *Egagropili*

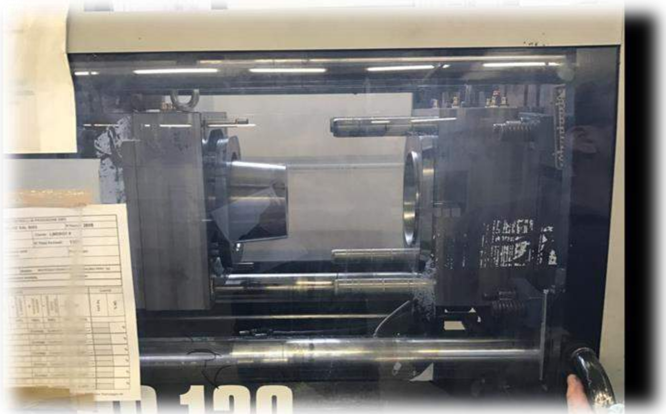


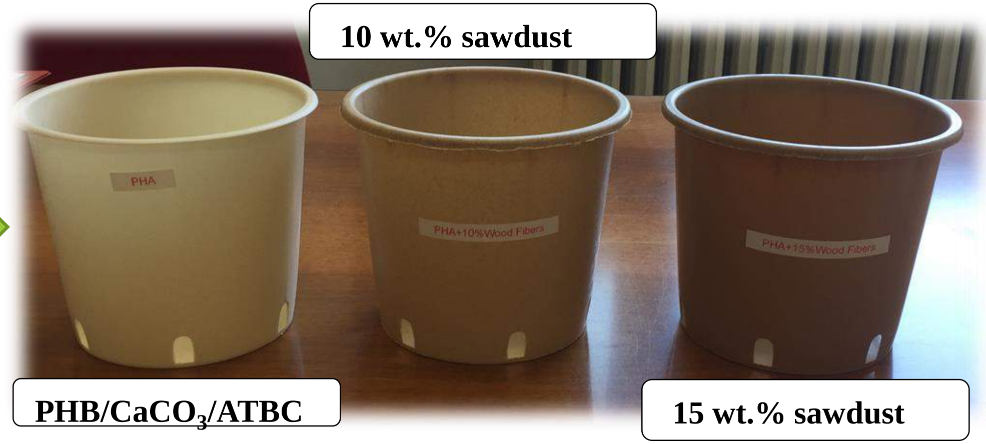


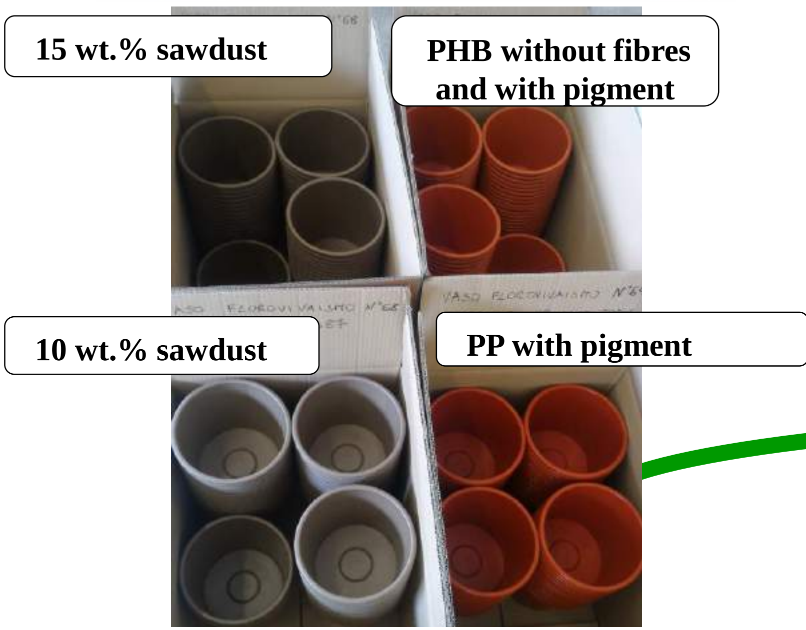
- ✓ **Pots, grids and tutors containing up to 15 wt.% *P. oceanica* fibres.**

to the aquaculture plant (Maricoltura s.r.l.) located at Rosignano Solvay (LI) for the **degradation/performance** tests of the produced items in **marine and dune mesocosms** and for the **performance tests of marine plants and psammopytes (dune plants)** grown in the produced pots.

POTS FOR "TERRESTRIAL" PLANTS BASED ON PHB AND SAWDUST



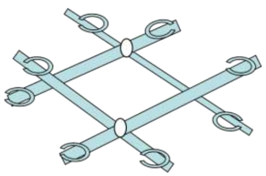




…. to a plant nursery located in Piombino (Livorno, Italy) for **degradation/performance** tests of the pots in different environments: **greenhouse**, **outside** and **underground** (buried).

Applications of products based on PHB and "egagropili": naturalistic engineering interventions

1. Restoration of seagrass meadows



Journal of Applied Ecology

Journal of Applied Ecology 2016, **53**, 567–578 doi: 10.1111/1365-2664.12562

Global analysis of seagrass restoration: the importance of large-scale planting

Marieke M. van Katwijk[1*], Anitra Thorhaug[2], Núria Marbà[3], Robert J. Orth[4], Carlos M. Duarte[3,5,6], Gary A. Kendrick[5], Inge H. J. Althuizen[1], Elena Balestri[7], Guillaume Bernard[8], Marion L. Cambridge[5], Alexandra Cunha[9], Cynthia Durance[10], Wim Giesen[1,11], Qiuying Han[12], Shinya Hosokawa[13], Wawan Kiswara[14], Teruhisa Komatsu[15], Claudio Lardicci[7], Kun-Seop Lee[16], Alexandre Meinesz[17], Masahiro Nakaoka[18], Katherine R. O'Brien[19], Erik I. Paling[20], Chris Pickerell[21], Aryan M. A. Ransijn

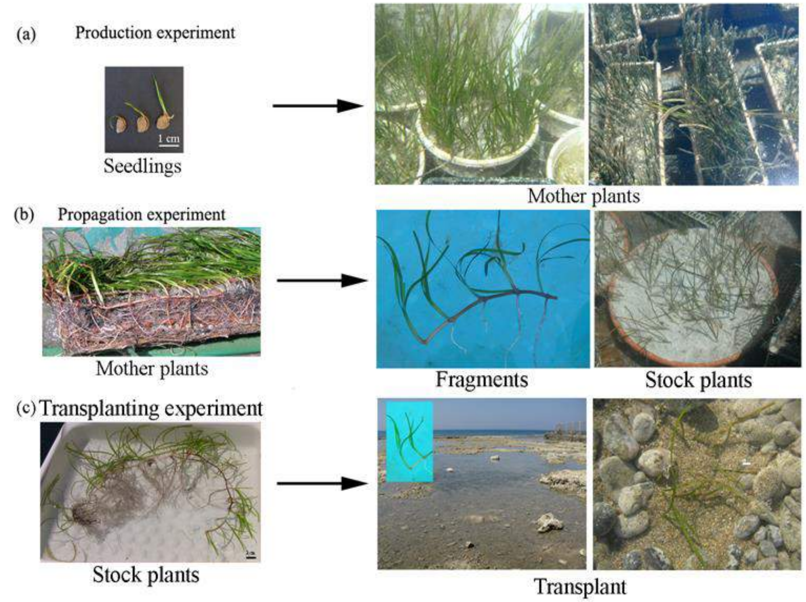
Journal of Applied Ecology

Journal of Applied Ecology 2012, **49**, 1426–1435 doi: 10.1111/j.1365-2664.2012.02197.x

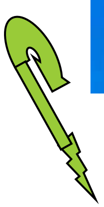
Nursery-propagated plants from seed: a novel tool to improve the effectiveness and sustainability of seagrass restoration

Elena Balestri* and Claudio Lardicci

Dipartimento di Biologia, Pisa University, Via Derna 1, 56100, Pisa, Italy



2. Restoration of coastal dune systems



Estuarine, Coastal and Shelf Science

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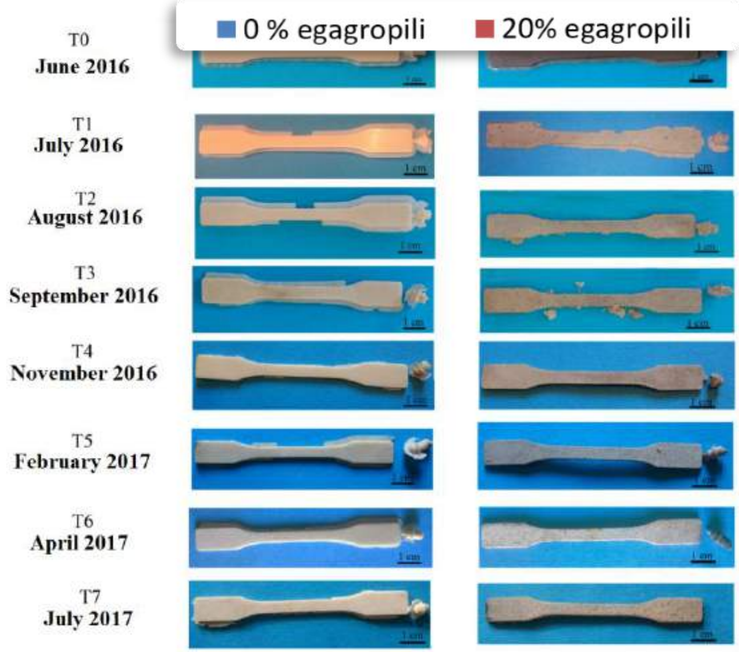
Application of plant growth regulators, a simple technique for improving the establishment success of plant cuttings in coastal dune restoration

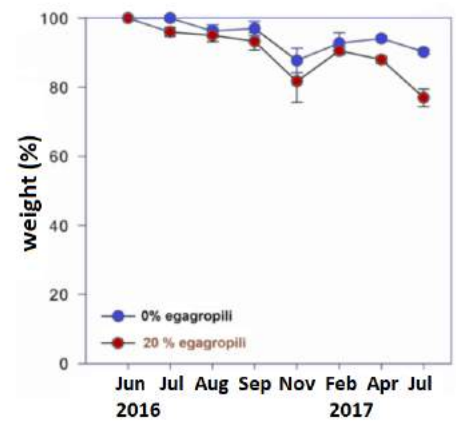
Elena Balestri*, Flavia Vallerini, Alberto Castelli, Claudio Lardicci

Dipartimento di Biologia, University of Pisa, Via Derna 1, 56126 Pisa, Italy

Degradation test in marine sediments (mesocosms)

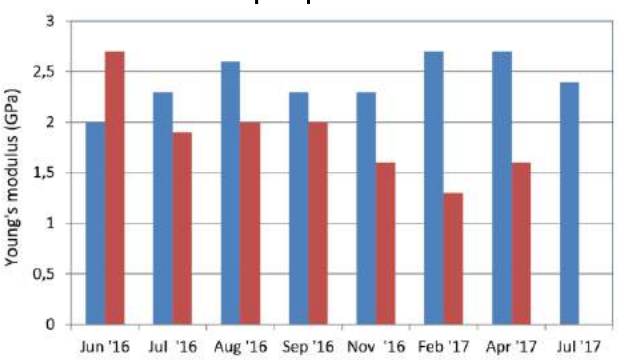
1. Weight loss (%) and changes of the tensile properties over the time

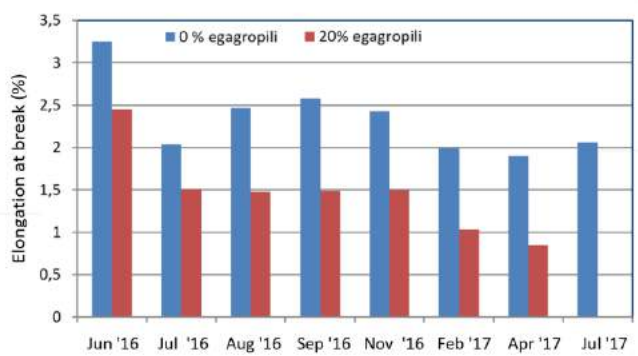




The composite with 20 wt.% PO fibres showed a marked reduction of the mechanical properties

…. after 16 months it was not possible to evaluate its mechanical properties



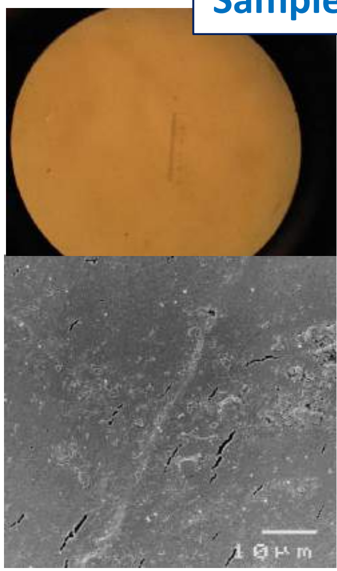


Degradation test in marine sediments (mesocosms)

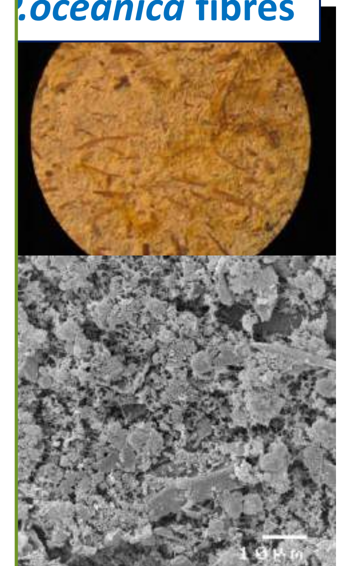
2. Development of a biofilm on the surface of "dog bone" specimens and morphological changes observed ... ope (SEM)

Sample w

.oceanica fibres



Time 0



after 3 months

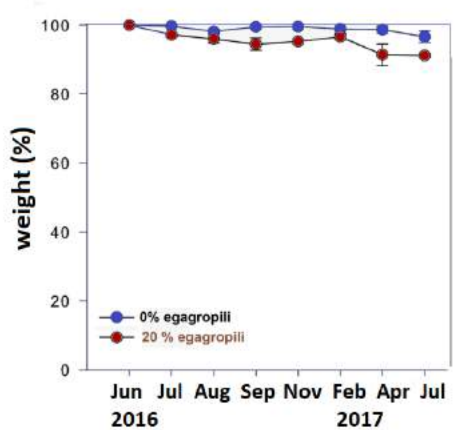
Evident biofi ... r 3 months

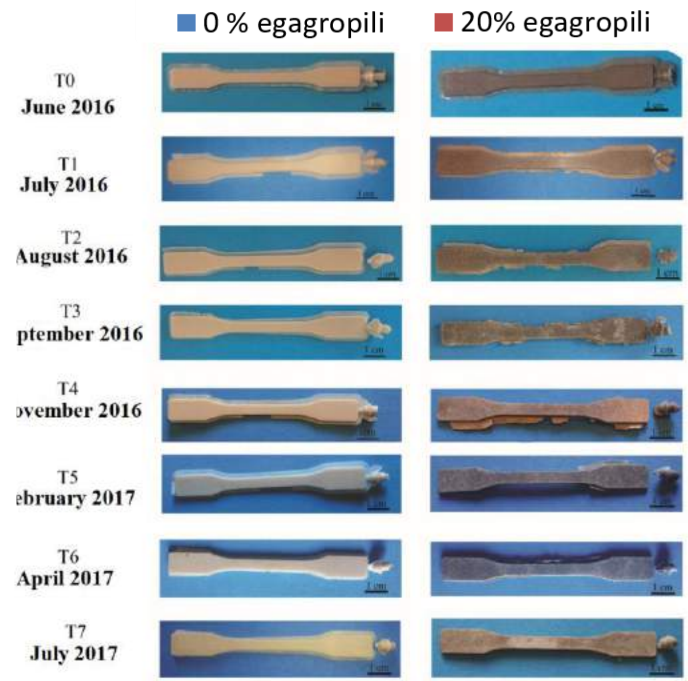
Pot based on **PHB** and **15 wt.% *P.oceanica* fibres after 15 months** in marine mesocosms.

Evident presence of a biofilm,
physical deformation but the pot resulted **intact**.

Degradation test in sand dune (mesocosms)

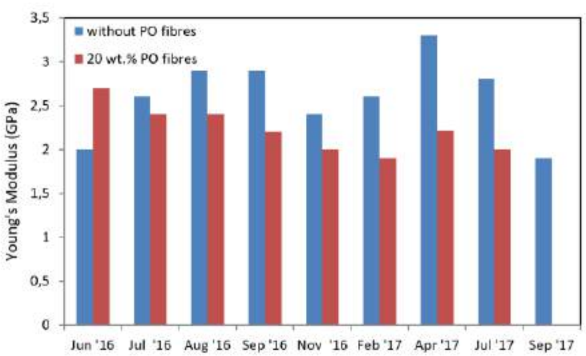
1. Weight loss (%) and change of the tensile properties over the time

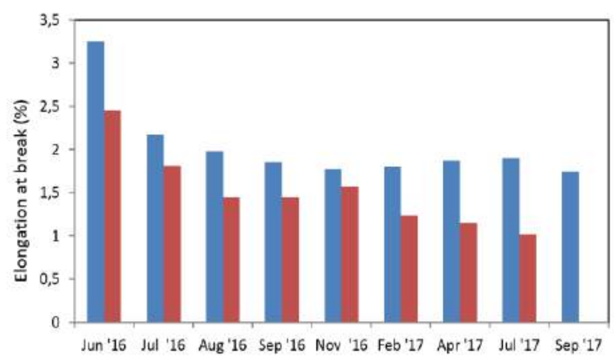


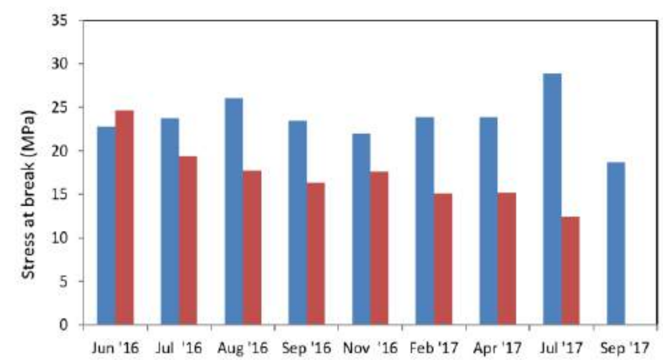


The composite with 20 wt.% PO fibres showed a more marked reduction of the mechanical properties

…. after **18 months** it was not possible to evaluate its mechanical properties



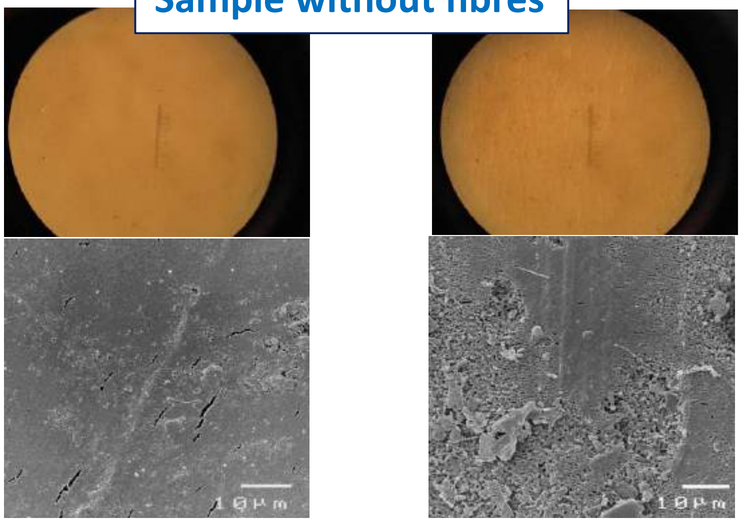




Degradation test in dune mesocosms

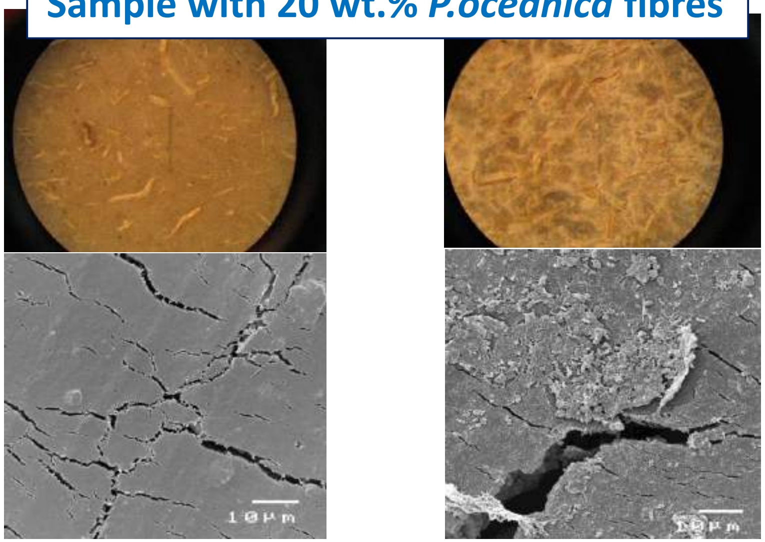
2. Development of a biofilm on the surface of "dog bone" specimens and morphological changes observed by stereomicroscope and scanning electron microscope (SEM)

Sample without fibres



Time 0 | **after 3 months**

Sample with 20 wt.% *P.oceanica* fibres

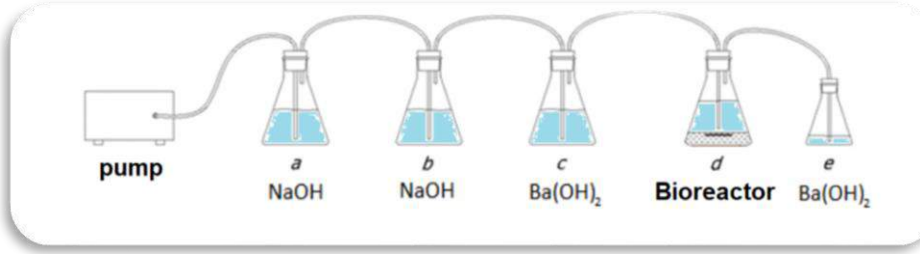


Time 0 | **after 3 months**

Degradation state more pronounced in the presence of P.oceanica fibers

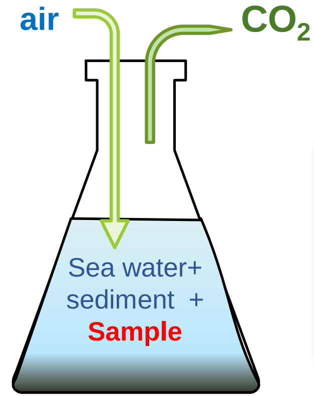
Biodegradation test in bioreactors containing sea water/marine sediment

1. Sea water/marine sediment collection from natural environment and biodegradability apparatus set-up



2. Measurement of evolved CO_2

Samples

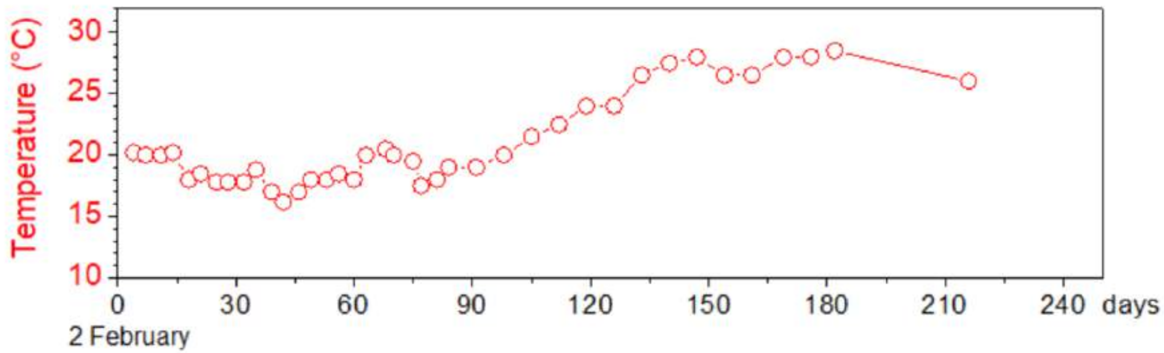


CO_2 evolved → % Biodegradability

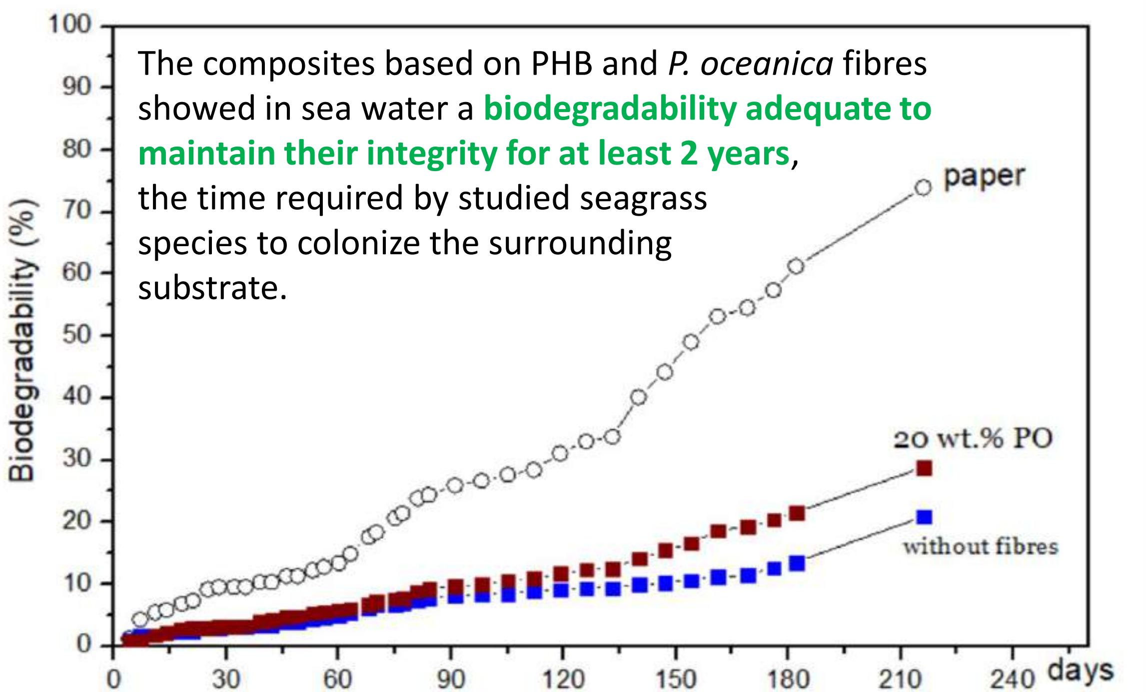
$$Biodegradability\ \% = \frac{\sum mgCO_2 - \sum mgCO_{2,blank}}{ThCO_2} \cdot 100$$

3. Quantitative and qualitative characterization of the microbial community responsible for degradation

Sediment removal at time 0 and at the end of experimentation, DNA extraction and sequencing.



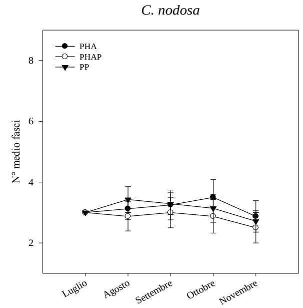
The composites based on PHB and *P. oceanica* fibres showed in sea water a **biodegradability adequate to maintain their integrity for at least 2 years**, the time required by studied seagrass species to colonize the surrounding substrate.

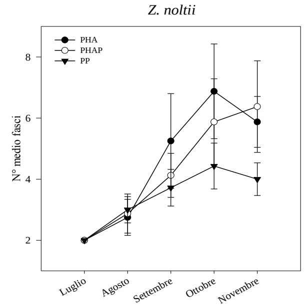


Valuation of the effects of the pots on the growth of marine and dune plants and microbial communities

1. Plant growth monitoring





✓ **Better growth performance** in pots based on PHB and PHB/PO fibres than in PP pots.

All composite pots show **initial signs of degradation** but are **still intact** after 15 months.

2. Qualitative and quantitative characterization of the microbial community of the sediment and rhizosphere (in progress…)



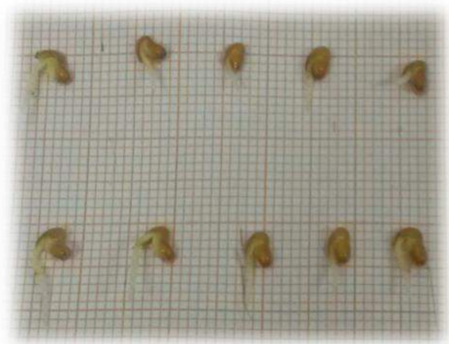

Composites based on PHB and sawdust fibres

Phytotoxicity by Germination test of *Lepidium sativum* L. seeds

The composite pellets were crushed to increase the surface/mass ratio and the resulting powders were extracted with deionized water (1:10 w/v) for 24 h using a horizontal shaker.
The eluates were used as germination medium.

$$GI(\%) = \frac{G_s \cdot L_s}{G_c \cdot L_c} \cdot 100$$

Adequate aeration in the dark for 42 h at 24°C



G_s and G_c are the number of seeds germinated for the sample and for the control, respectively;
L_s and L_c are the mean root lengths for the sample and for the control, respectively.

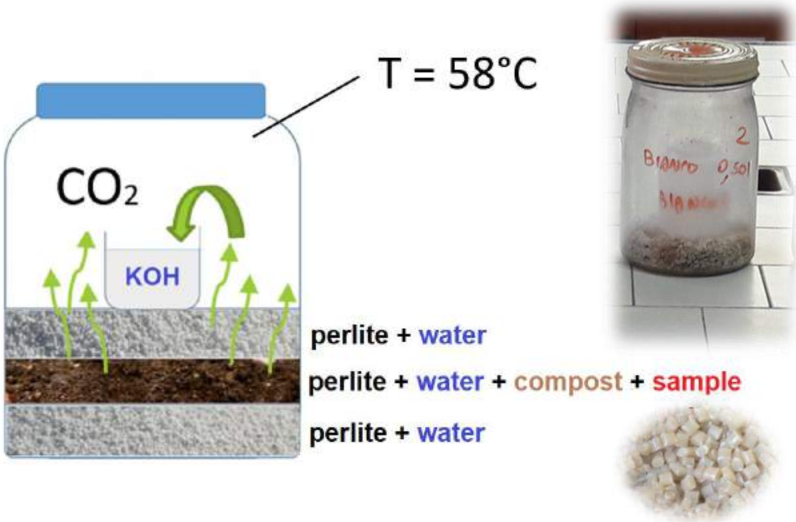
Germination Index	*GI (%)*	
	Eluate 100%	Eluate 75%/water 25%
Pigmented PP	**99.6**	**97.1**
PHB without fibres	**105.3**	**133.8**
PHB + 10 wt.% fibres	**95.0**	**129.4**
PHB + 15 wt.% fibres	**96.7**	**132.2**

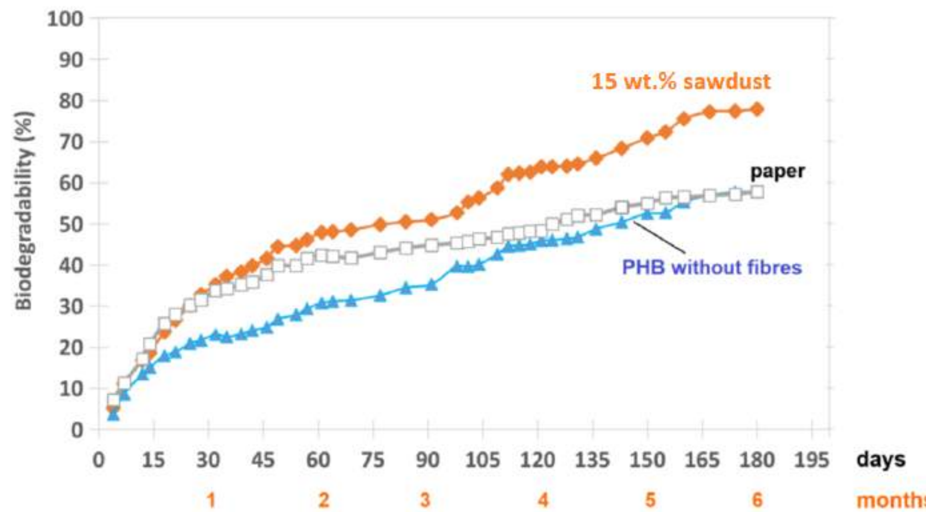
✓ GI(%) values above the threshold limit (60%) normally considered safe for water extracts obtained from compost (DL 75/2010)

the developed composites **are not phytotoxic**

Biodegradability/composting of the composites based on PHB and sawdust fibres

Mineralization test in compost (ASTM D5338-98)





CO_2 evolved → % Biodegradability

$$Biodegradability\ \% = \frac{\sum mgCO_2 - \sum mgCO_{2,blank}}{ThCO_2} \cdot 100$$

✓ After about 6 months the composite with **15 wt.% of sawdust fibres** showed a biodegradability of about **80 %** (**higher than paper**).

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Biodegradability/composting of the composites based on PHB and sawdust fibres

Disintegration test in compost (ISO 20200-2004)

After 90 days

Sample	*wt. % fibres*	*n.*	*m_i (g)*	*m_f (g)*	*Disintegration %*	*Average disintegration %*
PHB/ $CaCO_3$/ATBC	**0**	1	2.6720	0.3423	87.2	**92.6** > 80*
		2	2.7265	0.1544	94.3	
		3	2.5315	0.0909	96.4	
PHB/ $CaCO_3$/ATBC	**10**	1	2.5107	0.4161	83.4	**93.2** > 80*
		2	2.6675	0.0000	100	
		3	2.7589	0.1037	96.24	
PHB/ $CaCO_3$/ATBC	**15**	1	2.5518	0.0943	96.3	**94.2** > 80*
		2	2.5393	0.2892	88.6	
		3	2.5593	0.0594	97.7	

*limit value considered acceptable

Biodegradability/composting of the composites based on PHB and sawdust fibres

Mineralization and Disintegration tests in compost (ASTM D5338-98, ISO 20200-2004)

The developed **PHB and PHB/sawdust composites** resulted **compostable** in accordance with the European norm EN 13432:2000.

The presence of **sawdust fibres have accelerated the biodegradation** of the composites in compost, promoting the disintegration of the sample.

Performance of the "terrestrial" pots

Valuation in a plant nursery in three different enviroments

Crassula argentea

in greenhouse

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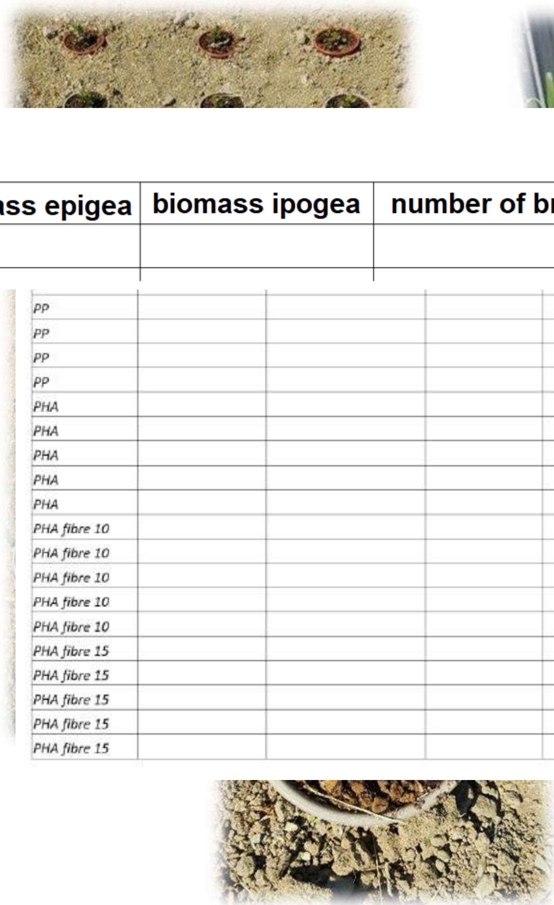
Performance of the "terrestrial" pots

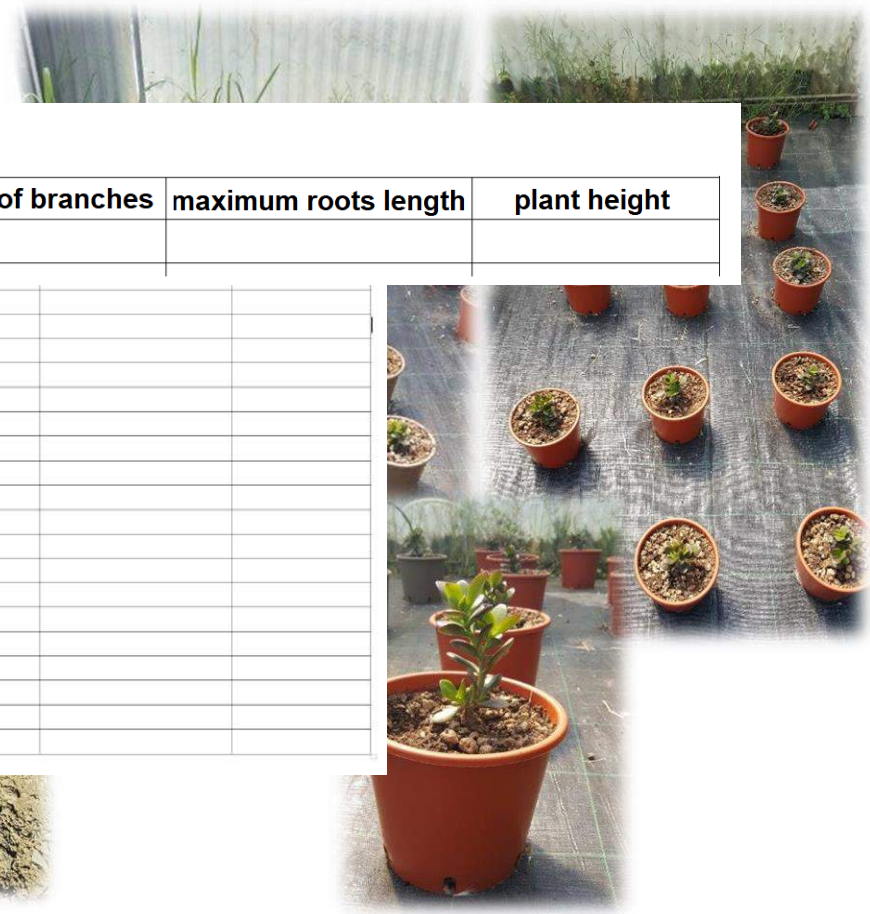
Monitoring of the pot performance and their effect on plant growth through control protocols

outside

buried



greenhouse



Date:

sample	biomass epigea	biomass ipogea	number of branches	maximum roots length	plant height
PP					
PP					
PP					
PP					
PP					
PHA					
PHA					
PHA					
PHA					
PHA					
PHA fibre 10					
PHA fibre 10					
PHA fibre 10					
PHA fibre 10					
PHA fibre 10					
PHA fibre 15					
PHA fibre 15					
PHA fibre 15					
PHA fibre 15					
PHA fibre 15					

Performance of the "terrestrial" pots

… after 6 months ….

outside

All pots **resulted intact** and showed **no evident** signs of **degradation.**

The **plant growth performance was the same** in all pots, independently of the pot type (PHB, PHB/PO, PP).

Performance of the "terrestrial" pots

…… after 6 months

greenhouse

All pots **resulted intact** and showed **no evident** signs of **degradation.**

The **plant growth performance was the same** in all pots, independently of the pot type (PHB, PHB/PO, PP).

Performance of the "terrestrial" pots

… after 6 months

Buried pots

PP pot

Buried PP pots showed **no evident** signs of **degradation.**

Pot without fibres

✓ Buried PHB-based pots showed **evident** signs of **degradation**, especially those containing 10 – 15 wt.% of sawdust……

confirming the results of the compostability tests.

Pot with 15 wt.% fibres

In conclusion …

what comes from the sea returns to the sea and what comes from the land returns to the land

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Thank you for attention