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Title: Biodegradability under marine conditions of bio-based and petroleum-based polymers as substitutes of conventional microbeads

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28 **Abstract:**

29 Plastic microbeads have been produced at very large scales since several decades for cosmetics
30 and personal care products, a large amount of which being directly transported in the oceans.
31 To better understand their biodegradability in marine environment and evaluate their possible
32 replacement by biodegradable polymers, seven polymer types were studied including three
33 conventional petroleum-based polymers (PE, PMMA and PCL), two bio-based polymers (PLA
34 and PHBV) and two natural products (rice seeds and apricot kernel). We used several
35 innovative approaches by both the experimental design and the set of multidisciplinary
36 techniques to follow the successive steps of biodegradation together with abiotic degradation
37 under seawater conditions. Congruent signs of biodegradability were observed by oxygen
38 consumption, weight loss, and modification of molecular weight, ¹H NMR and mass
39 spectrometry profiles. We found that microbeads made of PHBV, PCL, rice and in a lesser
40 extent apricot were biodegradable under our conditions, while not for microbeads made of PE,
41 PMMA and PLA. Taking into consideration of industrial application, PHBV could be the ideal
42 material to replace the conventional microbeads, since the material is resistant to hydrolysis,
43 but susceptible to microbial degradation, as shown by the ¹H NMR results. This study provides
44 the first time arguments for the use of biodegradable substitutes to replace conventional
45 microbeads to support the recent legislative rules aiming to reduce the pollution by primary
46 microplastics in the oceans.

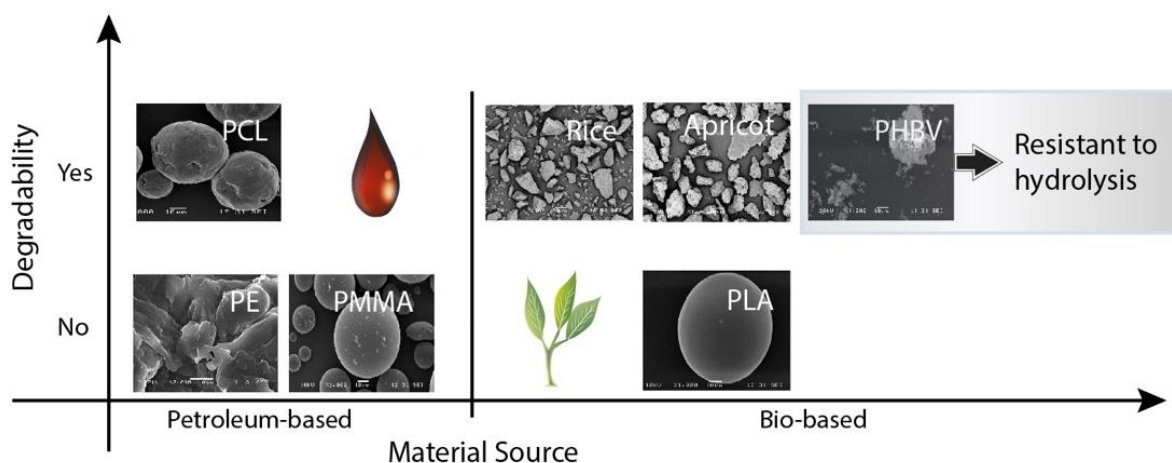
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Keywords: microbeads, biosourced and biodegradable polymer, cosmetics, plastic pollution, microbial ecotoxicology

Highlights:

- The biodegradation of 7 polymers in the marine environment was investigated to challenge their application as potential substitutes of the conventional microbeads used in cosmetics and personal care products.
- Innovative 2-stepwise experiments were designed to mimic realistic marine conditions.
- The four biodegradation steps were characterized, including biodeterioration, biofragmentation, bioassimilation and mineralization.
- The biosourced PHBV is proposed as the best biodegradable polymer tested to replace the conventional microbeads.

Graphical abstract:



1. Introduction

Accumulation of plastics in the marine environment has been observed in ocean gyres, on beaches and in sediments worldwide [1]. The major source of plastic at sea arises from mismanaged plastic waste and from riverine inputs [2]. Beside entanglement and ingestion of macro debris by large vertebrates (birds, turtles etc.) [3], adverse effects on marine organisms were particularly due to microplastics with proven transfer along the food chain [4]. Within microplastics, distinction has been made between small microplastics (25 μm –1 mm) that can be as abundant as large microplastics (1–5 mm) in the oceans [5]. Another distinction has been made between “primary microplastics” purposefully manufactured to be of millimeter size (e.g. microbeads and plastic pellets) and “secondary microplastic” that result from breaking down of larger plastic pieces due to UV light, waves or abrasion [6].

Microbeads are primary microplastics commercially available in particle sizes ranging from 10 μm to 1 mm, which are used in hundreds of products, often as abrasive scrubbers, including face washes, body washes, cosmetics, and cleaning supplies [7]. After use, microbeads included in personal care products are poured down the drain and cannot be collected for recycling. Wastewater treatment plant is generally not a sufficient solution to prevent microbeads pollution [8]. Microbeads from personal care and cosmetics products could be a significant source of microplastics pollution in the ocean [8–11]. It has been estimated that about 8 trillion microbeads were emitted into aquatic habitats per day from the United States [7].

As an alternative way to relieve the problem, it has been also advised to replace the conventional microplastic, generally made of polyethylene (PE) or polymethyl methacrylate (PMMA) by

83 'biodegradable' microparticles. Biodegradable plastics can be petroleum-based polymers such
84 as polycaprolactone (PCL), or bio-based polymers (so-called 'biosourced') such as poly(D-
85 lactide) (PLA) and polyhydroxyalcanoates (PHAs). Other natural compounds such as rice seeds
86 and apricot kernels have already been used as exfoliating material by some manufacturers and
87 are considered as bio-based polymers [12].

88 Most of scientific studies on plastic biodegradation were conducted under controlled laboratory
89 conditions by using soil or compost, but very few studies investigated the aquatic media and
90 especially the marine environment [13–15]. By now, few standard methods could be applied to
91 assess the biodegradability of plastics under marine conditions, but several scientists underlined
92 the drawbacks and biases of these methods [16–18]. Several authors underlined the need of
93 convergent results from combined approaches, including proof of mineralization by
94 microorganisms together with tests of chemical structure alteration in order to conclude on the
95 biodegradability of a polymer [19,20]. Another concern is the necessity to mimic biological,
96 physical and chemical conditions of the marine environment during the tests.

97 The aim of this study was to test the biodegradability under marine conditions of bio-based
98 (PLA, PHBV, apricot kernels and rice seeds) and petroleum-based polymers (PCL), as
99 substitutes of conventional microbeads (PE, PMMA) for cosmetics. We used microbeads of 50
100 to 250 µm diameter, which were commercially available or laboratory-made by solvent
101 emulsion-evaporation technique. Biodegradability tests were performed in a two-steps protocol
102 including a first 2-months incubation of each polymer type with natural seawater communities,
103 followed by a transfer of the plastic together with its natural microbial biofilm to a marine

minimum medium with no other carbon source than the plastic for another 2-months. We hypothesized that bio-sourced polymers may have a greater biodegradation capability compared to petroleum-based polymers under marine conditions. Here, we combined different methodological approaches including weight loss, continuous biological oxygen consumption (optical fiber luminescent sensor), changes in molecular weight (high temperature size exclusion chromatography), size and surface properties (laser diffraction particle size analyzer and scanning electron microscopy), carbonyl index (Fourier transform infrared spectroscopy) and release of oligomers (^1H nuclear magnetic resonance spectroscopy and Liquid Chromatography-High Resolution Mass Spectrometry).

2. Materials and Methods:

2.1. Production of petroleum-based and bio-based microbeads

The petroleum-based microbeads PE and PMMA were supplied by Good Fellow company (Lille, France). Natural microparticles were manufactured by cryogrinding of the rice seeds (Rice Exfoliator 200, Lessonia, Saint Thonan, France) so called “rice” hereafter and apricot kernels (Apricot Exfoliator 200, Lessonia, Saint Thonan, France) so called “apricot” hereafter. PE, PMMA, rice and apricot were sieved in order to recover the microparticles for which the diameter was ranging from 50 to 250 μm .

PCL (CAPA 6800, Perstorp Company, Sweden), PLA (7001D, Nature Works, Blair, USA) and PHBV (ENMAT Y1000P with a HV molar ratio of 3%, Tianan Biological Materials Co. Ltd., China) were obtained as pellets and transformed in spherical microbeads by solvent emulsion-

evaporation technique (see Method S1 and Table S1 for material preparation of PCL, PLA and PHBV).

2.2. Microparticle characterization

Microparticle surface morphologies were observed at the step of microbeads production by using a Jeol JSM-6031 scanning electron microscope (SEM). Prior to any observation, the fracture surfaces were coated with a thin gold layer by means of a Polaron sputtering apparatus.

2.3. Experimental setup

A two phases stepwise experiment was designed in order to evaluate the biodegradability of the conventional petroleum-based and bio-based polymers under marine conditions (Fig. 1). The first step consisted in the formation of a mature biofilm in natural seawater, as previously described [21,22]. Briefly, each microparticle type (called “microbeads” hereafter) was incubated for a period of 2-months in 1.8L aquarium (Sodispan, Spain) with direct circulation to the sea. Seawater was pumped at 14 m depth in the Banyuls Bay close to the SOLA observatory station of the Laboratoire Arago (SOLA station - NW Mediterranean Sea). A flow rate of 50 ml.min⁻¹ was chosen to ensure a sufficient renewal of natural bacteria (every 30 min) and the surface outlet water was filtered at 50 µm. Each aquarium contained 12 grams of each microbead types (PE, PMMA, PCL, PLA, PHBV, apricot and rice) added for a 2-months period. Throughout the experiment, seawater temperature (between 18.3°C and 25.3°C) and salinity (38.5) in the aquarium were similar to seawater from the Banyuls bay.

Second, about 10 mg of dry microbeads were transferred under sterile conditions to 4.9 mL closed glass vials (Interchim, Montluçon, France) containing 2 mL of minimum medium (called

“MM” hereafter) with microbeads as sole carbon source (see Method S2 for MM composition). Vials were incubated in the dark at 18°C under agitation at 110 rpm (orbital agitator) for a period of 2 months (called “biotic condition” hereafter). In addition, controls were incubated and sampled in the same way and consisted in triplicate vials containing 2 mL of MM with microbeads of the same composition but previously sterilized overnight in 70% ethanol and evaporation under UV sterile hood (called “abiotic condition” hereafter). A total of 130 vials were needed to follow the different parameters detailed below in triplicate samples taken after 0,1, 3, 7, 15, 30 and 60 days of incubation (Fig. 1).

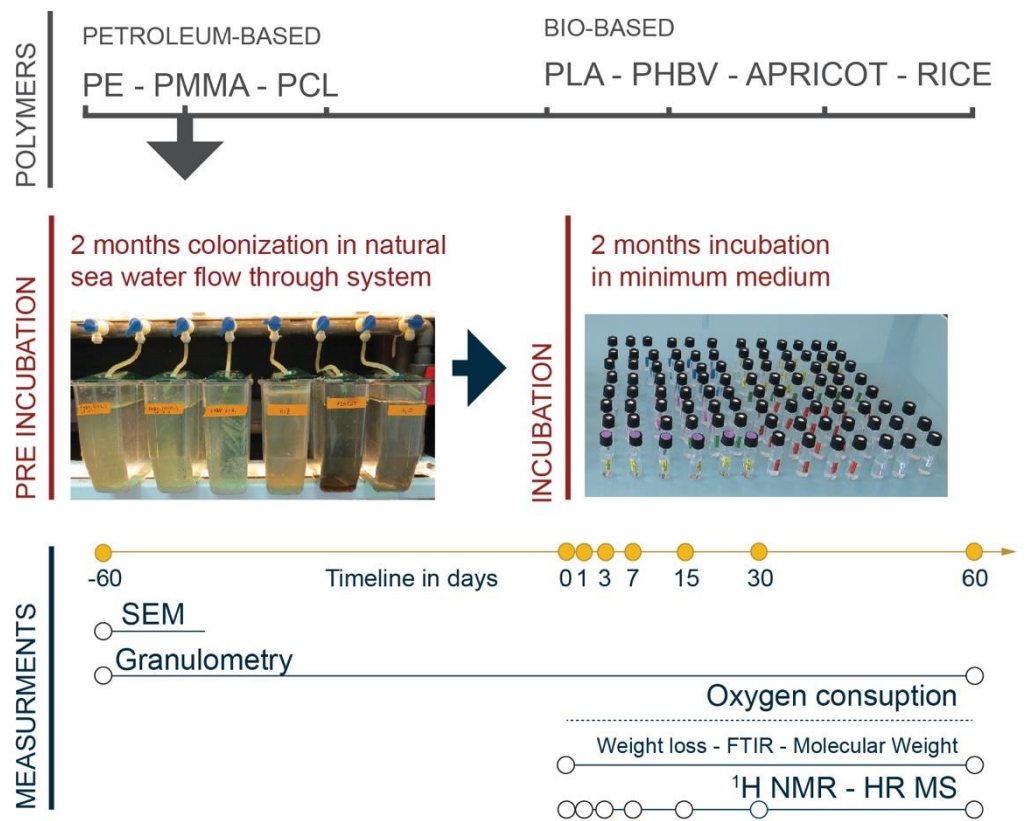


Fig. 1 Experimental design. Schematic presentation of the two phases stepwise experiment of (1) a 2-months incubation in natural seawater flow through system (flow rate 50 mL.min⁻¹) for petroleum-based (PE, PMMA, PCL) and bio-based microbeads (PLA, PHBV, apricot, rice) followed by (2) a transfer into 4.9 mL glass vials containing 2 mL minimum medium with microbeads as sole carbon source for another 2-months. Lines indicate when measurements were possible for respective assays and dots indicate each measurement done during the course of the experiment, dashed line indicates daily oxygen measurement.

162

163 2.4. Continuous oxygen measurement and biodegradation

164 During the second step of the experiment (Fig. 1), triplicate vials with each microbead type
165 were equipped with an optical fiber luminescent oxygen sensor (Presens, SP-PSt3-YAU) using
166 the instrument of Stand-alone Fiber Optic Oxygen Meter (Fibox 4, Germany). Oxygen sensors
167 were placed in the liquid phase to obtain the concentration of dissolved oxygen. Oxygen
168 concentration was manually measured daily in the abiotic (PE and PHBV) and biotic conditions
169 in minimum medium and oxygen was converted from mole concentration to total oxygen in
170 vial with the Henry's law (oxygen equilibrium confirmed between the two phases) and ideal
171 gas law. In the case of PHBV, vials were opened under a sterile laminar flow hood after 15 days
172 to ensure that oxygen was not limiting for bacterial growth and closed again. In this case,
173 oxygen concentration was always maintained $> 15 \mu\text{mol.L}^{-1}$ and the re-opening for 10 minutes
174 was enough to return to initial values (around $230 \mu\text{mol.L}^{-1}$). For all polymer types, biological
175 oxygen demand (BOD) was computed from the difference of oxygen concentration. The
176 consumption of oxygen in abiotic condition served as a negative control. The percentage of
177 biodegradation was expressed as the ratio of the oxygen consumption over the theoretical
178 amount of consumed oxygen for complete degradation (ThOD), as previously described [23].

179 2.5. Size change

180 The size distribution of the microparticles were measured at the beginning (*D-60*) and at the
181 end of the experiment (*D60*) (Fig. 1) with a laser diffraction particle size analyzer (Malvern
182 Mastersizer 2000 model with a Scirocco 2000 module) through granulometry analyses. The

particles were analyzed according to a model of spherical particles of polystyrene and according to the Fraunhofer diffraction theory [24]. Each measurement was an average of 15 scans and each sample was analyzed, thus providing the number, volume and length of the particles.

2.6. Weight loss

Microbeads weight was measured on *D0* and *D60* of the incubation in MM in triplicates (Fig. 1). The pellet was washed twice with distilled water to remove salts with 1 min centrifugation at 13000 rpm, then lyophilized and immediately weighed in analytical balance with the precision of 0.1 mg (ENTRIS1241-1S, Sartorius).

2.7. Molecular weight

Molecular weight was determined for each polymer before colonization and at the end of the incubation in MM (Fig 1). High temperature size exclusion chromatography (HT-SEC) analyses were performed using a Viscotek system (Malvern Instruments) equipped with a combination of three columns (Polefin 300 mm × 8 mm I. D. from Polymer Standards Service, porosity of 1000 Å, 100 000 Å and 1 000 000 Å). Samples were dissolved in the mobile phase (tetrahydrofuran) with a concentration of 5 mg mL⁻¹ and 200 µL of sample solutions were injected and eluted in tetrahydrofuran using a flow rate of 1 mL min⁻¹ at 25 °C. The same procedure was applied to polyethylene samples using 1,2,4-trichlorobenzene at 150 °C to dissolve the sample and as mobile phase. The mobile phase was stabilized with 2,6-di(tert-butyl)-4-methylphenol (200 mg L⁻¹). Online detection was performed with a differential refractive index detector, a multichrom right angle light scattering detector, and a viscometer detector. A universal calibration curve, obtained with polystyrene standards (Polymer Standards

Service, Mainz, Germany) in the range of 0.5–7.106 kg mol⁻¹, was used to calculate the molar mass distribution of the samples. OmniSEC software version 5.12 (Malvern Panalytical, UK) was used for the calculations. The typical evaluated accuracy for this technique is close to 10%.

2.8. Fourier Transform Infrared (FTIR) spectroscopy

Fourier Transform Infrared (FTIR) analyses were performed on microbeads on *D0* and *D60* of the incubation in MM in biotic condition (Fig. 1). After collection, the supernatant was removed and samples were dried at 60° in an aerated oven until constant weight to eliminate trace of water. FTIR measurements were carried out using a Nicolet™ 380 FT-IR (ThermoFisher Scientific) in ATR (Attenuated Total Reflectance) mode. For each sample, the total amount of microbeads (10 mg or less) was compressed against a diamond crystal with a constant force. FTIR spectra were collected using 16 scans from 4000 cm⁻¹ to 400 cm⁻¹, with a resolution of 4 cm⁻¹. To assess the variation between spectra, the characteristic absorption bands were realized using Omnic Spectra software (Thermo Fisher Scientific). For each polymer, manual assessment of absorption band was done and compared to the literature. The calculations for carbonyl and crystallinity index are detailed for each polymer in the supporting material (Table S2-8).

2.9. Characterization of the degradation products by ¹H NMR spectroscopy

¹H NMR spectroscopy analysis were carried out to assess the release of degradation products (oligomers) from microbeads as sole carbon source for the biofilms incubated in minimum medium for 0, 1, 3, 7, 15, 30 and 60 days in abiotic and biotic condition (Fig. 1). For each point, samples (microbeads + liquid media) were transferred to 2 mL Eppendorf tube and centrifuged to remove the microbeads. Supernatants (540 µl) were supplemented with 60 µL of a 2 mM

solution of TSPd₄ (sodium tetra deuterated tri methylsilyl propionate, Eurisotop) in deuterated water (D₂O, Eurisotop). D₂O signal was used for locking and shimming while TSPd₄ constituted a reference for chemical shifts (0 ppm) and quantification. ¹H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, equipped with a 5 mm inverse-triple tuned (TXI) ¹H/¹³C/¹⁵N with z-gradient coil probe (Bruker Biospin Wissenbourg, France), with 5 mm-diameter tubes containing 600 µl of sample. 128 scans were collected (90° pulse, 3.24 s acquisition time, 4.0 s relaxation delay, 4789.272 Hz SW, 65536 data points). Water signal was eliminated by pre-saturation. An exponential filter was applied before Fourier transformation, and a baseline correction was performed on spectra before integration. Under these conditions, the limit of quantification is in the range of 0.01 mM. The relative quantity of oligomers was expressed using the TSPd₄ as the reference.

2.10. Liquid Chromatography- High Resolution Mass Spectrometry

When detected by NMR spectroscopy, the degradation products were then identified by High Resolution Mass Spectrometry. The extracts were analyzed using Electrospray Ionization (ESI) with a Q-Exactive Orbitrap™ mass spectrometer (Thermo Scientific) providing a mass accuracy lower than 3 ppm and an ultra-high resolution over 100 000. Briefly, 5 µl of supernatant from extracted samples were analyzed using positive and negative ion mode. The ion source and the capillary heater were set to operate at 3.2 kV and 320 °C. Nitrogen gas was used for nebulizing and as the damping and collision gas in the mass analyzer.

3. Results

3.1. Temporal changes of microparticle sizes

The SEM images of the raw particles showed significantly different size, sphericity and roughness properties depending on how the material was obtained (Fig. 2). The production of microbeads by cryo-grinding increased the roughness and the size distribution of particle compared to the solvent emulsion-evaporation technique. Size distribution estimated by granulometry for PLA, PCL and PHBV microparticles was centered approximately 100 μm (median diameter between 88 and 126 μm depending on the types of bioplastics) (Table 1). The PE microparticles were the largest with a median diameter around 280 μm . On the other hand, those made of PMMA were the smallest since their median diameter is around 60 μm . Natural microparticles made from rice or apricot had much more heterogeneous dimensions over a wide range of sizes confirming SEM observations. The median diameter for these microparticles was estimated to be around 120 and 160 μm , respectively.

After 2-months of incubation in natural seawater followed by 2-months incubation in minimum medium, sample aggregation was observed for most samples except for PHBV. No change of the size distribution was observed for the PMMA. For PLA and PE, values of the median diameter were similar. For rice, median diameter was higher after 4 months, with no smaller particles. For PCL, apricot and PHBV (Fig. S1), decreases of the median diameters were observed with the decrease of bigger particles.

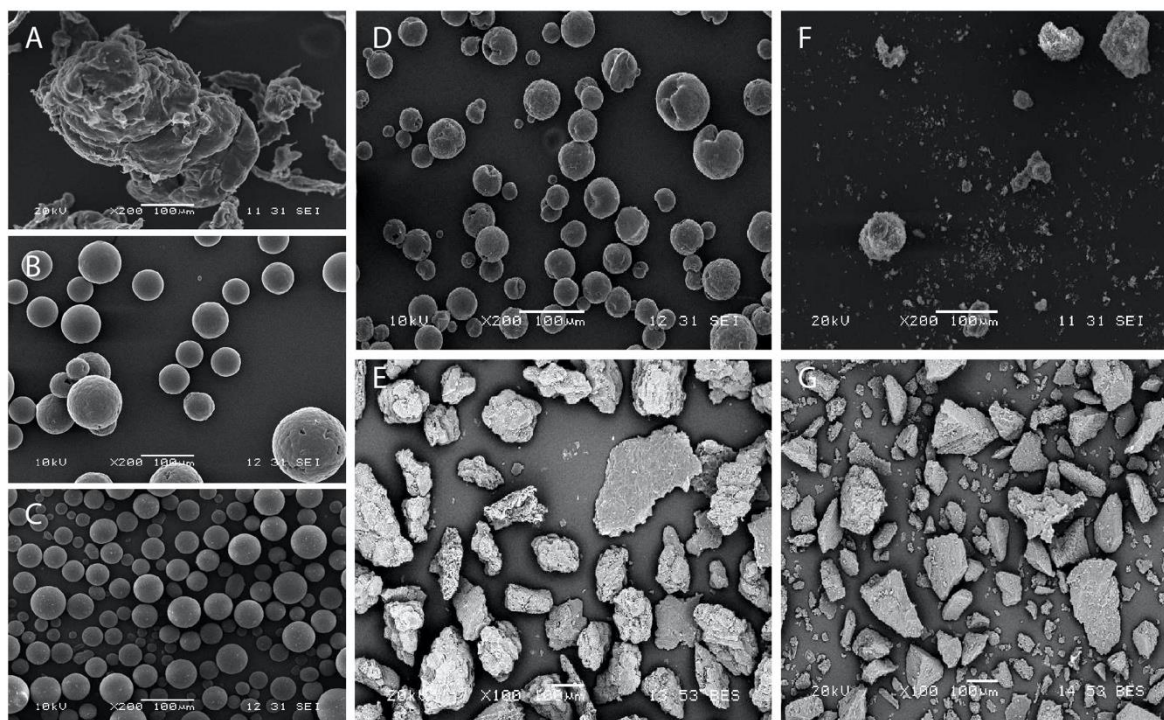


Fig. 2 Scanning electron microscopy for the microbeads made of seven polymer types: (A) PE, (B) PLA, (C) PMMA, (D) PCL, (E) ground apricot kernels, (F) PHBV, (G) ground rice particles. The scale bar in all panels are 100 μm .

3.2. Temporal dynamic of oxygen consumption

The dissolved oxygen was recorded daily for the first 15 days for the triplicates. No oxygen consumption was observed for abiotic control, as illustrated by PE and PHBV abiotic conditions in Fig. 3A. In biotic condition, the continuous evolution of oxygen consumption by bacteria in minimum medium with each polymer as sole carbon source showed different patterns according to the polymer types. Polymers including PE, PMMA and PLA did not show any sign of oxygen consumption. On the contrary, a drastic decrease of 19 μmole was observed for PHBV during the first 10 days, PCL, Rice and Apricot also showed significant oxygen consumption of 11.1, 7.3, and 2.4 μmole for the same period, respectively (Fig. 3B). The biological oxygen demand was then calculated for the first 10 days, before the first opening of the vials to avoid oxygen

limitation for PHBV in biotic conditions. being aware of the possibility of oxygen limitation for PHBV. The percentages of biodegradation were $4.1 \pm 0.4\%$ (average and standard deviation), $1.0 \pm 0.1\%$, $3.8 \pm 1.9\%$ and $0.5 \pm 0.2\%$ for PHBV, PCL, rice and apricot, respectively. It has to be noted that vials with rice became visually very turbid, which may affect the transmission of luminescence and then underestimate the oxygen consumption and resulting percentage of biodegradation. The two different oxygen consumption patterns have clearly classified the materials into two groups: the degradable and non-degradable ones (Fig. 3).

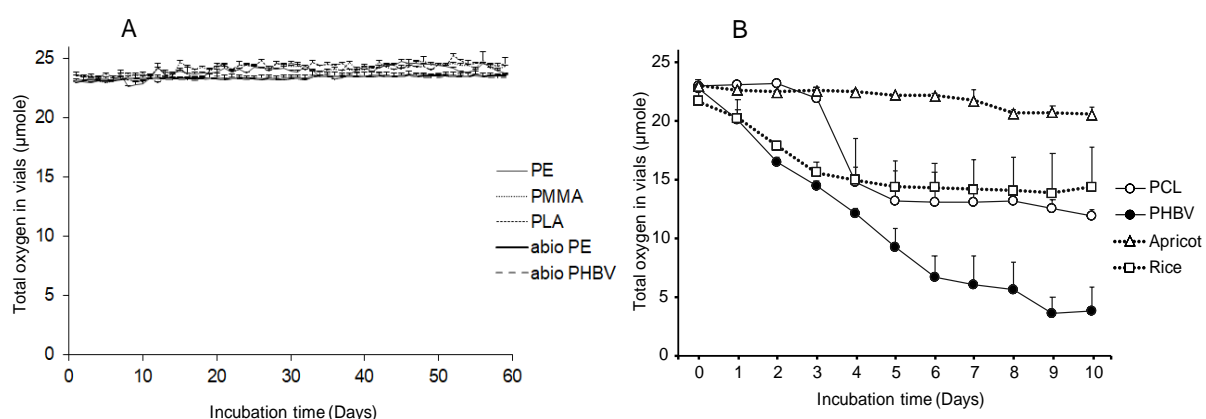


Fig. 3 Oxygen consumption (in μmole) after incubation in minimum medium with microbeads made of the seven material types under abiotic (abio PE and abio PHBV) and biotic (with their respective biofilm on PE, PMMA, PLA – left ; PCL, PHBV, apricot, rice - right) conditions (with standard deviation).

Type	Polymer	Monomer	Median diameter (μm)		Biodegradation rate % (S.D.)	Weight loss %	Molecular weight				Carbonyl */ Crystallinity index		¹ H NMR Signal	Mass Id
			D-60	D60			D0	Mn D60	Mw/Mn D0	Mw/Mn D60	D0	D60		
Conventional	Low Density Polyethylene (PE)		278	282	n.d.	0%	5200	5700	8.9	9.1	0*	0*	n.d./ n.d.	n.a.
	Poly(methyl methacrylate) (PMMA)		57	56	n.d.	0% (25.5%)	125 000	118 000	2.6	3.0	n.a.		n.d./ n.d.	n.a.
	Polycaprolactone (PCL)		88	61	1.0% (0.1%)	17.8% (7.2%)	33 000	23 000	1.7	2.0	7.57* /0.38	4.24* /0.53	n.d./ ++	Caprolactone (and its dimer and trimer)
Biosourced	Poly(lactic acid) (PLA)		115	119	n.d.	-0.3% (18.6%)	66 270	66 250	1.7	1.7	n.a.		n.d./ n.d.	n.a.
	Poly (3-hydroxybutyrate-co-3-valerate) (3 mol % HV) (PHBV)		126	106	4.1% (0.4%)	17.0% (6.1%)	34 0000	n.a.	n.a.		0.74	0.83	+ / n.d.	3-hydroxybutyric acid 3-hydroxyvaleric acid 3-hydroxybutyrate dimer 3-hydroxybutyrate-3-hydroxyvalerate dimer 3-hydroxyvalerate dimer (and its Heterotrimers)
Natural polymer	Crushed Apricot kernel (APRICOT)		158	120	0.5% (0.2%)	5.9% (8.8%)	n.a.		n.a.		n.a.		n.d./ ++	derivative compounds from ligno-cellulosis and oil constituents (carbohydrates, lignin and fatty acids)
	Crushed rice seed (RICE)		120	277	3.8% (1.9%)	80.1% (4.8%)	n.a.		n.a.		n.a.		n.d./ ++	derivative compounds from protein and starch constituents (carbohydrates, amino acids)

3.3. Weight loss

Weight loss measurement on *D0* and *D60* of the incubation in MM separated the polymers in 2 groups similar to those obtained by oxygen measurement (Table 1). No weight loss was found on polymer for PE, PMMA and PLA. Apricot showed $5.9 \pm 8.8\%$ of weight loss, when PCL, PHBV showing about $17.8 \pm 7.2\%$ and $17.0 \pm 6.1\%$. Rice had the highest weight loss with $80.1 \pm 4.8\%$.

3.4. Changes in the molecular weight

Significant signs of degradation were only observed for PCL that showed a decrease by 30% of average molecular weight (M_n) (from 33 000 to 23 000 g mol⁻¹) and an increase of their polydispersity index (1.7 to 2.0) that indicates a higher molecular weight dispersion (Table 1). No significant change was found for PE, PMMA and PLA microbeads. Measurement of the molecular weight was not applicable for the, PHBV, rice and apricot.

3.5. Modification of the chemical properties

The composition of microbeads incubated under biotic conditions was controlled by FTIR after 2 months of incubation in MM. No biodegradability could be found for PE, PMMA and PLA (Fig. S2-4). Moreover, the absence of characteristic carbonyl band at 1720 cm⁻¹ indicate that PE microbeads did not undergo photodegradation.

The FTIR spectra of PCL microbeads showed a decrease of carbonyl index and a concomitant increase of crystallinity index (Table1, Fig. S5). Such pattern is typical of ester bonds cleavage in amorphous region of the polymer chains and confirms the reduction of the molecular weight of the polymer. Rice microbeads presented a spectrum characteristic of starch with specific

monosaccharides and polysaccharides signals (Fig. S6). The strong modification of the spectra over time revealed a transformation of starch constituent amylopectin, amylose into simple monomer of glucose (Fig. S7). The FTIR analysis of apricot microbeads presented a typical spectrum of lignocellulosic material. The spectra of these sample did not change over time, thus indicating a low transformation of the material as observed by weight loss measurements (Fig. S8). An increase of the crystallinity index was observed for PHBV samples, suggesting the possible cleavage of ester bonds in amorphous region of the polymer chains (Table 1, Fig. S9).

3.6. Products release during the biodegradation

Under abiotic conditions, ^1H NMR signal was detected only in the supernatant of PCL, rice and apricot samples incubated in minimum media while no signal was recorded for the other materials (Fig. 4A). This indicate the release of small molecular weight compounds from these polymer matrixes in the aqueous medium when no biological process occurs. The extraction of oligomers quickly started after incubation. The integration of the total NMR signal clearly showed a gradual release of oligomers during the 60 days of incubation (Fig. 4A). By comparison with the literature data, the signal attribution confirms the identification of short chains of PCL oligomers and typical signal of carbohydrates such as glucose was observed for the oligomers extracted from rice and apricot samples (Fig. S10 and Table S9). It is worth to notice that oligomers from PCL, rice and apricot were not detected under biotic conditions, suggesting an assimilation concomitant to their production. On the contrary, the PHBV microbeads did not release any measurable oligomers under abiotic conditions but a signal was recorded in presence of microorganisms, thus confirming the biotic degradation of polymers

(Fig. 4B). The release of oligomers (3-hydroxyvalerate, 3-hydroxybutyrate repeating unit) started slowly after one day of incubation with microorganisms and increased up to 30 days. The signal then decreased until the end of experiment which is typical of microbial consumption.

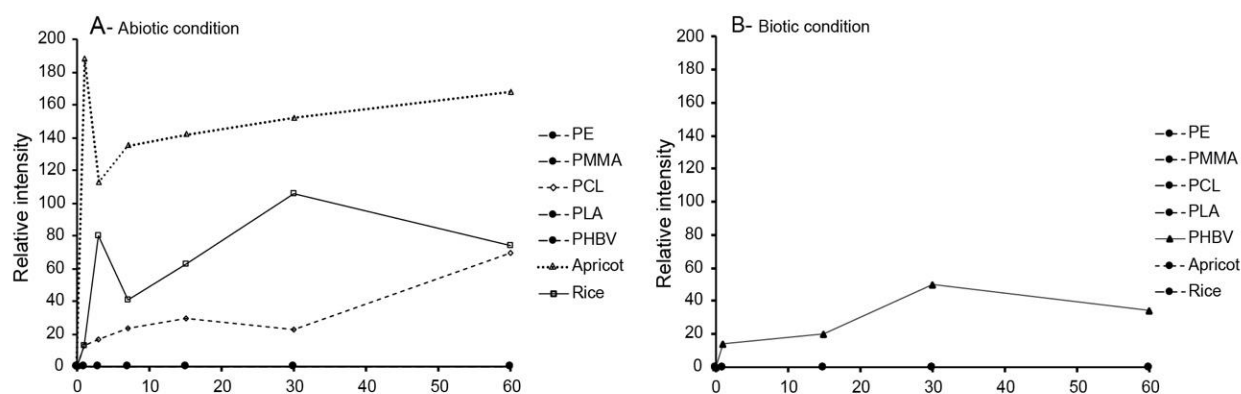


Fig. 4 Production of oligomers over 60 days of incubation in minimum medium with microbeads made of the seven material types (PE, PMMA, PCL, PLA, PHBV, apricot, rice) under (A) abiotic (without biofilm) and (B) biotic (with biofilm) conditions. The results represent the total integration of ^1H NMR spectra expressed in relative intensity of TSPD₄ internal standard.

3.7. LC Orbitrap *TM* Mass Spectrometry

The oligomers detected in the supernatant were analyzed by mass spectrometry to identify their molecular composition. Analysis of PCL, rice and apricot supernatants confirmed the presence of small molecular weight compounds corresponding to the abiotic degradation of the polymer matrix into various size of oligomers (Fig. S11 and Table S10). Under abiotic conditions, the ester hydrolysis of PCL generated caprolactone oligomers that were identified as monomer, dimer and trimer of polycaproic acid oligomers. As well, the mass spectra obtained from rice and apricot supernatant provided a complex profile of low molecular weight compounds. Carbohydrates and amino acids were detected in rice samples while degradation products from lignin and fatty acids were identified in apricot samples, resulting from the transformation of

the raw material by abiotic processes. Finally, the PHBV microbeads incubated with microorganisms released various dimer/trimer units of hydroxy-butyrate and hydroxy-valerate. In our conditions, PHBV microbeads were more sensitive to enzymatic cleavage than to abiotic degradation processes.

4. Discussion

4.1. An original multidisciplinary approach for the estimation of polymer biodegradability in the marine environment

Standard test procedures for estimating polymer biodegradability are commonly based on measurements of biological O₂ demand or CO₂ consumption, which are giving indication on the complete biomineralization of the material by microorganisms [17]. In this study, we combined the standard test together with other methodologies in order (i) to confirm the standard test results based on biomineralization processes and (ii) to give more insights on the other steps involved in biodegradation that include also the biodeterioration, biofragmentation and the bioassimilation of the polymers [25]. Physico-chemical and structural modification of the microbeads and evidence of biodeterioration were assessed by granulometry, gravimetry and FTIR spectroscopy and size exclusion chromatography, ¹H NMR and high-resolution mass spectrometry were performed to identify degradation products (monomers and oligomers) in biotic and abiotic conditions and then give information on the biofragmentation. Finally, ¹H NMR analysis also showed the bioassimilation of the polymers. To our knowledge, this is the first time that molecular analysis was used to identify the biofragmentation products (i.e.

oligomers) and their bioassimilation in polymer degradation by complex natural microbial communities. Here, we propose that NMR spectroscopy and mass spectrometry provide new and highly informative results on the molecular fragments of the polymers that can be generated during its biodegradation. Moreover, it brings a complementary information on direct assimilation of polymers by microorganisms, that are missing parameter in the classic methodological approaches which focus either on the growth of microorganisms or on the loss of material properties, thus addressing the biodeterioration with indirect processes.

While there is no harmonized indication today for the preparation of the microbial inoculum to test its polymer biodegradation capabilities, convergent views indicated that incubation with pre-selected isolated strains, as it is specified for regional standard tests [26,27], do not mimic the large diversity of microorganisms living on plastics in the natural conditions, the so-called 'plastisphere' [28,29]. Complex natural marine inocula were adopted by two international standards [30,31], where it is unfortunately indicated that the inocula could be stored for a month at 4 °C, which drastically increase the possibility of a strong modification of the plastic-degrading community [32]. Another novelty of our study is to propose an original two-steps protocol to test the polymer biodegradability by a natural mature biofilm. The first step of formation of the mature biofilm was performed by incubating the polymer types in natural seawater [21]. The second step is the biodegradability test, where the polymers with its mature biofilm were transferred into a minimal medium with no other carbon source and energy. This procedure is classically used to test the capability of microorganisms to degrade various organic

compounds or pollutants [33,34], but this is the first time that the complex biofilm community has been used for polymer degradation under marine condition.

Finally, another originality of our study is that no other feasibility study exists ranging from the bio-based and biodegradable microparticle design to the evaluation of its fate at sea. Most of the biodegradation study so far were using commercially plastics, mainly in the form of film [17,15] and to a less extent in powder [35,36], rarely in the form of microbeads. The successful use of cryogrinding and solvent emulsion-evaporation techniques showed that such approaches may be useful to conduct further studies on other polymer types or for the production of biodegradable substitutes to replace conventional microbeads [37–39].

4.2. Successive steps of biodegradability by complex marine bacterial communities for bio-based and petroleum-based polymers

Our multidisciplinary approach provides the first evidence of the successive steps involved in polymer biodegradation in seawater. These steps have been theoretically proposed by several authors [18], but never evidenced before by a complementary set of techniques on the same samples and by involving a complex mature biofilm grown under natural seawater conditions. Here, we have highlighted a group of polymers including PCL, PHBV, apricot and rice showing congruent signs of biodegradation, and another group made of PE, PMMA and PLA that gave no sign of biodegradation under our conditions. We prove here that PCL, PHBV, rice and apricot microbeads undergo a succession of physico-chemical transformation (as revealed by FTIR) that lead to the biofragmentation of the material (weight loss, molecular weight) resulting in the release of short length oligomers (mass spectrometry, ¹H NMR). Then, these

hydrosoluble compounds can be readily assimilated by the microorganisms (^1H NMR) and support the respiratory activity of the biofilm (BOD). This sequential process does not occur for PE, PMMA and PLA microbeads, as no or slight oxidation can be initiated in our experimental time frame.

Our results are congruent with other studies describing PHBV and PCL as biodegradable polymers under natural conditions in soil [40,41]. Other studies found no sign of biodegradation for PLA in freshwater [42]. PCL films were also found to present less biodegradability potential in freshwater than under marine condition, probably due to very different bacterial communities inhabiting these ecosystems [43].

Interestingly, we found that one of the bio-based polymers (PLA) did not fall into the group of biodegradable polymers and contrariwise, one of the petroleum-based polymers (PCL) showed clear signs of biodegradation. These counterintuitive results underline again that bio-based polymers does not necessary mean environmental degradable polymers, and inversely petroleum-based polymer does not necessary mean non-biodegradable products, as already mentioned elsewhere [16].

4.3. A set of congruent results for each polymer type

4.3.1. Biodegradation of PCL. We found that the weight loss for PCL was 17.8% under our experimental conditions. Besides, *Lu et al.* [44] found a weight loss around 18% for 2 month period in natural seawater environment. Oxygen consumption confirmed the presence of biodegradation, while an unusual behavior was observed for PCL, with a steep decrease of oxygen concentration after 3 days (Fig. 3), which could be attributed to the modification of

bacteria community and/or the availability of the dissolved oligomers. The release of oligomers in abiotic condition proved here that a slight hydrolysis of PCL occurred in seawater without any other biological attack, which did not be realized by some other tests [42]. There was no signal captured by ^1H NMR for PCL in biotic condition, indicating the rapid utilization of the oligomers released by abiotic processes and the presence of bioassimilation activity, which is consistent with other evidence of biotic degradation of PCL in seawater by others [43, 45,46]. Finally, the increase of crystallinity index from the FTIR results together with the reduction of molecular weight confirmed the degradability of PCL in seawater.

4.3.2. Biodegradability of PHBV. In our conditions, PHBV showed a weight loss of 17% (2 month and a biodegradability of 4.1% (10 days). The weight loss was also reported for PHBV with different degree of degradation. For instances, a study performed in natural seawater from coastal France showed relatively lower value, with the 30% weight loss for a period of 6-months for plastic film[35], the observed differences could result from the material size and shape. During this study, ^1H NMR assay showed oligomer release only in biotic but not in abiotic condition, thus making evidence of an efficient biofragmentation of PHBV by the marine bacteria which was under or beyond their capability to assimilate the large amount of oligomer produced. Thus, our research showed the stability of PHBV by the analysis of the released of the oligomers (^1H NMR). Abiotic degradation has not been observed under other seawater conditions within 6 months incubation at 25°C by weight loss assay [47]. In addition, negligible abiotic degradation (weight loss) was also found after 21 days under composting conditions at 60 °C [48]. Therefore, together with our results, PHBV could be described as quite resistant to

moisture or abiotic attack. We also found an increase of crystallinity index that showed the preference of the biofilm to attack amorphous region of the polymer, further proving previous described results [49–51].

4.3.3. Biodegradation of apricot and rice. It is noteworthy that it was the first study which performed the degradation test on the two natural materials in the marine environment. Both the results of oxygen consumption and ^1H NMR confirmed its degradability in marine conditions. Under abiotic condition, carbohydrates such as glucose was gradually released from rice and apricot, and the released soluble carbohydrate fraction was readily assimilated by the microorganisms. Previous studies on rice straw also showed a high biodegradation rate in the soil and freshwater environments [52,53].

4.3.4. Other polymers. No sign of biodegradation was detected by our set of complementary techniques for PE, PMMA and PLA. Beside their chemical structures and properties (carbon-carbon bonds and/or crystallinity), the environmental conditions (relatively low temperature, salinity, low nutrients and others) may also be a cause of limited degradation in seawater [54,55]. The lack of obvious oxidation of these polymers limit their fragmentation, the production of intermediate products (< 1500 Da) and thus their assimilation by microbes in marine environment [18,56,57].

Our multidisciplinary techniques gave congruent results for the polymer biodegradability, except for the aggregation problem from granulometry results probably due to the formation of extracellular polymeric substances after the bacterial colonization [58]. It has been noticed that PCL, rice and apricot could gradually release the oligomers, thus the weight loss measurement

of the three polymers could be overestimated. With the complementary techniques, such as oxygen consumption and ^1H NMR results, etc. the three materials showed biodegradable in the marine environment.

5. Conclusions

An increasing number of countries have implemented laws to restrict the production of microbeads over the world. We present here that, out of the seven materials, microbeads made of PHBV could be good candidate for substitution of conventional microplastics classically made of PE or PMMA, since the material is quite resistant to hydrolysis and susceptible to microbial degradation, as proved from this study.

Our study presented several novelties regarding its experimental design but also by the various and complementary technologies used. We advise here that not only respirometry tests classically used in standard tests, but rather a multidisciplinary approach reflecting the 4 steps of biodegradation should be considered for further studies dealing with the biodegradability of polymers in the environment. It includes other banned single-used plastics such as plastic bags, straws, disposable dishes, cotton swabs (such as Q-tips) and coffee stirrers.

We also underlined that clear distinction should be made with bio-based polymers that may not be biodegradable (such as PLA), when less sustainable substitute materials showed clear signs of biodegradation at sea (such as the petroleum-based PCL). Finally, further studies are needed to evaluate the toxicity of the polymers and their degradation products, as well as the release of additives generally supplemented by manufacturers in the microplastic composition.

502 ***Declaration of Competing Interest***

503 The authors declare that they have no known competing financial interests or personal
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505

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511

512 ***Appendix A. Supplementary data:***

513 Supplementary methods including polymer preparation with solvent emulsion-evaporation
514 method, minimum medium composition and detailed information on molecular weight and ¹H
515 NMR measurements; supplementary results including spectra and spectra descriptions for FTIR
516 results on the seven polymers, for ¹H NMR spectra and ESI-MS mass spectra of PCL, rice,
517 apricot and PHBV.

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