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1 **Title: Marine biodegradation of tailor-made polyhydroxyalkanoates (PHA)**
2 **influenced by the chemical structure and associated bacterial communities**

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20 **Keywords:** Polyhydroxyalkanoate (PHA), Biosynthesis, Biodegradation, Plastisphere

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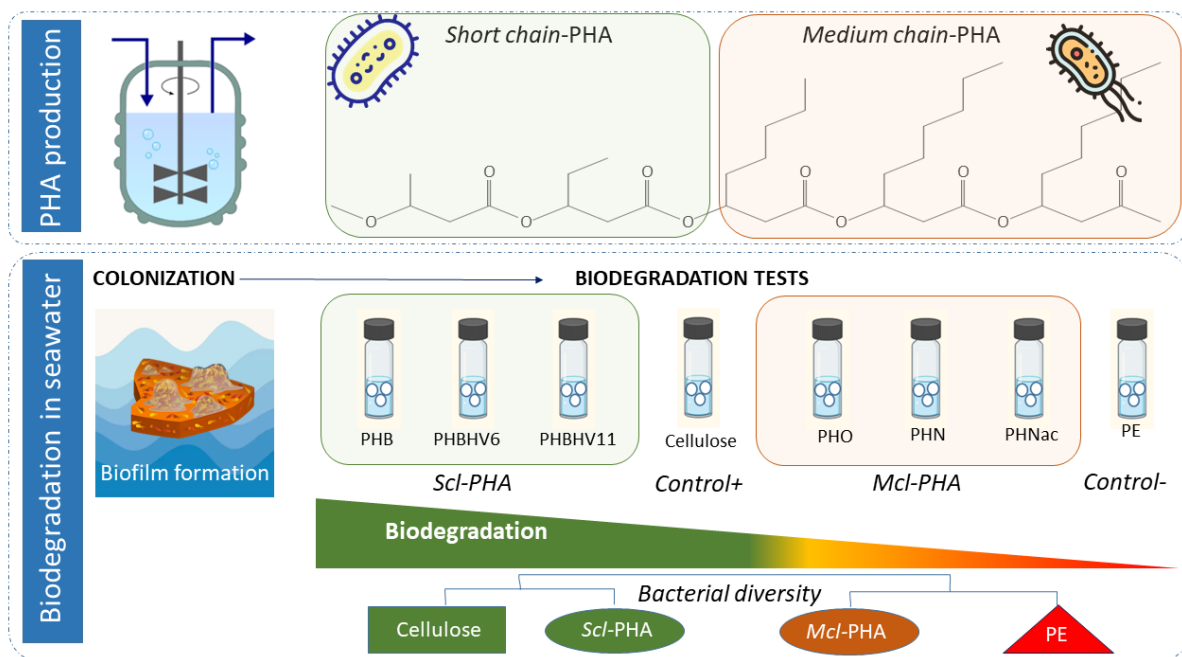
22 **Abstract**

23 Over recent years, biodegradable polymers have been proposed to reduce environmental
24 impacts of plastics for specific applications. The production of polyhydroxyalkanoates (PHA) by using
25 diverse carbon sources provides further benefits for the sustainable development of biodegradable
26 plastics. Here, we present the first study evaluating the impact of physical, chemical and biological
27 factors driving the biodegradability of various tailor-made PHAs in the marine environment. Our

28 multidisciplinary approach demonstrated that the chemical structure of the polymer (i.e. the side chain
 29 size for *short-* vs. *medium-chain* PHA) which was intrinsically correlated to the physico-chemical
 30 properties, together with the specificity of the biofilm growing on plastic films (i.e. the associated
 31 ‘plastisphere’) were the main drivers of the PHA biodegradation in the marine environment.

32 **Environmental implication:**

33 Plastic pollution of the environment is a critical problem that has the potential for long-lasting
 34 impact. While all plastics eventually break down to at least some degree, they can remain in different
 35 transition states for extended periods of time, such as microplastics and nanoplastics, that represent
 36 different types of hazards. PHA currently occupy a growing portion of the biodegradable plastics
 37 market and relevant studies on their biodegradation are needed to address potential environmental
 38 problems.



39

40

41 **1. Introduction**

42 Plastic pollution is nowadays a global and evident environmental crisis that is of major
 43 concern to marine organisms (Deudero and Alomar, 2015), but also contributes to raise global
 44 greenhouse gas emissions and climate change (Shen et al., 2020). The marine environment constitutes
 45 a large reservoir of mismanaged plastic waste, with 4.8 to 12.7 million metric tons of plastic entering

46 the oceans every year (Jambeck et al., 2015). As a partial solution, it has been proposed to
47 manufacture plastics that would be both bio-based, i.e. made from renewable resources, and
48 biodegradable in a given environment (compost, soil, water) over a reasonable amount of time (weeks,
49 months). The biodegradable plastics have been considered relevant for selected applications with
50 respect to their use and end of life (Paul-Pont et al., 2023). Among the bio-based and biodegradable
51 polymers, polyhydroxyalkanoates (PHA) are considered as a promising alternative to fossil-based or
52 non-biodegradable polymers. Mainly but not only from bacterial origin, the PHA constitute a large
53 family and display a wide range of chemical compositions and properties according to the producing
54 strain, the source of carbon used for feeding and the fermentation process. PHA can be divided into
55 two subgroups: short chain-length PHA (*scl*-PHA) composed of monomers of 3 to 5 carbon atoms,
56 and medium chain-length PHA (*mcl*-PHA) composed of monomers of 6 to 14 carbon atoms. The
57 physico-chemical properties differ between the *scl*-PHA, that are rigid and brittle polymers and the
58 *mcl*-PHA that are usually more rubbery and ductile (Pérez-Rivero and Hernandez-Raquet, 2017).
59 Poly(3-hydroxybutyrate) (PHB) is one of the most widespread and best characterized among the PHA.
60 With high crystallinity (>50%), it is a relatively brittle and stiff polymer (Corre et al., 2012; Koller et
61 al., 2010). Production of the copolymer with valeric acid triggers the incorporation of 3-
62 hydroxyvalerate (HV) and results in the less stiff and brittle copolymer poly(3-hydroxybutyrate-co-3-
63 hydroxyvalerate) (PHBHV), easier to process for commercial applications (Lemechko et al., 2019;
64 Pachekoski et al., 2009). *Mcl*-PHA display properties that could replace elastomers. They are rubbery,
65 soft and show a lower degree of crystallinity, melting temperature and glass transition (Abe et al.,
66 2012). Despite the recent interest in their properties for biomedical or cosmetic applications, *mcl*-PHA
67 are not produced in industrial quantities and the relationships between their biodegradation and their
68 physico-chemical properties have been poorly explored (Abe et al., 2012).

69 Biofilm growing on plastic is characterized by very diverse and niche-specific microbial
70 communities called the “plastisphere” (Zettler et al., 2013) that can play a predominant role in plastic
71 degradation (Jacquin et al., 2019). Previous studies showed the great abilities of microbial
72 communities to biodegrade *scl*-PHA in the marine environment (Deroiné et al., 2014; Volant et al.,
73 2022). The microbial communities colonizing commercial PHBHV under marine conditions have been

74 studied during semi- and long-term colonization, but no clear relation was made with the rate of
75 biodegradation (Dussud et al., 2018; Odobel et al., 2021). Due to the limited commercial availabilities,
76 no studies have ever reported microbial activity and diversity on *mcl*-PHA, thus resulting in a lack of
77 comparison between the environmental end of life of plastics made of *scl*- or *mcl*-PHA families.

78 In this study, we describe the bacterial production of 6 different tailor-made *scl*- and *mcl*-PHA
79 by *Halomonas sp. SF2003* (Thomas et al., 2019) and *Pseudomonas putida KT2440* (DSM 6125),
80 respectively, together with their physico-chemical characterization. We also analysed their
81 biodegradation by using a two steps protocol including a one-month pre-colonisation step in a flow-
82 through aquarium with natural seawater for each PHA group (*scl*- and *mcl*-PHA) and controls
83 (cellulose and Polyethylene, PE) to mimic the growth of marine natural biofilms and another step in
84 minimum medium with plastics as sole carbon source to test biodegradation. We hypothesized that
85 various PHA types, related chemical and physical properties, as well as the associated natural
86 biofilms, are driving the biodegradation in seawater. We used a multidisciplinary approach to produce
87 (bioreactor) and characterize six tailor-made PHA (gas chromatography, steric exclusion
88 chromatography, contact angles, differential scanning calorimetry) and to evaluate the bacterial
89 diversity (16S rDNA Illumina sequencing) associated to the biodegradation (oxygen consumption,
90 heterotrophic bacterial production) of each polymer type.

91

92 [2. Materials and methods](#)

93 [2.1. Production of *scl*- and *mcl*-PHAs](#)

94 Pre-cultures of *Halomonas sp. SF2003* for *scl*-PHA production and *Pseudomonas putida*
95 *KT2440* for *mcl*-PHA production were both performed in 500 mL Erlenmeyer flasks at 30 °C and 200
96 rpm, with incubations for 8 h in Zobell media (Thomas et al., 2019) or for 16 h in mineral medium
97 (Maclean et al., 2008), respectively. The preculture was then transferred into a 5 L bioreactor (GPC-
98 BIO, MINIPROLAB, France) containing a final volume of 2 L of Zobell medium or mineral medium
99 for *scl*- and *mcl*-PHA production, respectively. Cultivation temperature was 30 °C, pH 7.0 ± 0.2.
100 Agitation was at a minimum of 400 rpm to maintain a dissolved oxygen concentration above 30 %, as
101 measured with optical dissolved oxygen sensors (Hamilton company, Switzerland).

102 Prior to PHA accumulation from different carbon sources, *scl*-PHA fermentation started with
103 10 g.L⁻¹ of glucose to promote growth. After 12 h, one pulse of 5 g.L⁻¹ of glucose was added every 4
104 hours until 24 h for the PHB accumulation. A mix of glucose and valeric acid (50/50, %mol) was
105 continuously dropped in the bioreactor at a rate of 4 mL.h⁻¹ for 24 h for the poly-(3-hydroxybutyrate-
106 co-3-hydroxyvalerate) (PHBHV6) fermentation. A mix of glucose and valeric acid (70/30, % mol)
107 was continuously dropped at a rate of 6 mL.h⁻¹ for 24 h for the PHBHV11 fermentation. *Mcl*-PHA
108 fermentation also started with glucose implementation to promote high cell density before PHA
109 accumulation, as previously described (Sun et al., 2006). Briefly, this included a first phase of 24 h
110 growth with a feeding strategy based on pulses of exponential quantity of glucose over 7 h (12.5 g.L⁻¹
111 of glucose) followed by a linear feeding strategy from 7 to 24 h by adding a pulse of 1 g of glucose
112 additionally to the previous quantity of glucose pulsed every hour. After 24 h, 1 g of fatty acids
113 (octanoic, heptanoic or nonanoic acid or a mix of nonanoic acid and acrylic acid) was added to
114 produce a poly(3-hydroxyoctanoate) (PHO), a poly(3-hydroxynonanoate) (PHN) and another PHN
115 called “PHNac”, respectively) was added when the dissolved oxygen concentration was above 30%
116 (approximately every 15 minutes).

117 At the end of the fermentation, bacterial cells were recovered by centrifugation and PHA were
118 separated from bacterial biomass using an incubation with an excess of solvent under stirring in a glass
119 bottle (50 mL of chloroform for approximately 1 g of *scl*-PHA at 60°C overnight and 40 mL of
120 dichloromethane at room temperature overnight for approximately 1 g of *mcl*-PHA). For *scl*-PHA
121 extraction, distilled water (V/V) was added after cooling. The suspension was mixed and centrifuged
122 to recover the organic layer before filtration on glass fiber cotton and casting in a glass Petri dish. *Mcl*-
123 PHA solution were filtered through a 1.2 µm glass microfiber and the concentrated *mcl*-PHA solution
124 was precipitated in cold ethanol (10% v/v) and stored at 6 °C for two days before collection. The
125 casting process consisted of dissolving PHA in their respective solvents, pouring PHA solutions into a
126 glass Petri dish covered with lids that were opened briefly twice a day until constant weight to allow
127 slow solvent evaporation. Films were stored at room temperature and in the dark during three weeks
128 before any characterizations. Thickness of the films ranged from 80 to 120 µm for all PHA films.

129

130 2.2. Physico-chemical characterization of the six tailor-made PHA films

131 Compositions of the produced PHA were determined by gas chromatography (GC). *Scl*-PHA
132 and *mcl*-PHA underwent a propanolysis (Riis and Mai, 1988) and a methanolysis (Furrer et al., 2007),
133 respectively. Briefly, 10 mg of PHA were dissolved in 1 mL of chloroform or dichloromethane and
134 further propyl esterified (*scl*-PHA) with 1 mL of a solution of 1-propanol/37% HCl (8/2, V/V) or
135 methyl esterified (*mcl*-PHA) with 1 mL of MeOH/BF₃ (10% Boron trifluoride, V/V) at 80°C for 20 h.
136 After cooling down, distilled water (V/V) was added and the solutions were vortexed. The organic
137 phase was retrieved, dried on MgSO₄, filtered on glass fiber cotton and samples were injected on a
138 Perkin Elmer Clarus 480 gas chromatograph equipped with a 30 m x 0.32 mm DB-5 column (HP)
139 with splitless injector and flame ionization detector (FID). Oven temperature, ramp and nitrogen flow
140 were measured according to Riis and Mai (1988) and Furrer et al. (2007).

141 Molecular weights were measured by steric exclusion chromatography (SEC) using an Agilent
142 Technologies 1200 Infinity II containing an isocratic pump, a column oven at 35 °C and a RI detector.
143 PHA sample separations were performed by two columns PLgel (Mixed-E, 3 µm and Mixed-D, 5 µm)
144 from Polymer Laboratories for *scl*-PHA and by two columns from Malvern Panalytical technologies
145 (LT4000L, 4µm and LT5000L, 10 µm) for *mcl*-PHA and a column guard. About 10 mg of PHA were
146 first dissolved into 2 mL of chloroform (*scl*-PHA) or THF (*mcl*-PHA) then filtered with PTFE filter
147 (0,45 µm) before a 50 µL injection. The calibration was done with polystyrene standards from Agilent
148 Technologies.

149 Contact angles were measured on each PHA films using a drop shape analyser from KRÜSS
150 scientific technologies with a 2 µL droplet of water placed on the top surface-air side of the solvent-
151 cast films that have been previously stabilized for 3 weeks at room temperature. More than 5
152 measurements were carried out for a single sample and the resulting values were averaged.

153 Thermal properties were determined by differential scanning calorimetry (DSC) using a
154 Mettler-Toledo DSC-882 equipment. About 6 mg of PHA were taken from the cast films of PHA.
155 Particular attention was given so that all the PHA samples experienced the same thermal history (3
156 weeks ageing at room temperature in the dark for all solvent-casted films) in order to avoid the

157 induction of different structures, as reported elsewhere (Crétois et al., 2016; Laycock et al., 2014; Xie
158 et al., 2008). PHA samples were equilibrated to 25°C then heated to 190 °C for *scl*-PHA or 80°C for
159 *mcl*-PHA at 10 °C.min⁻¹ and kept isothermal for 2 min followed by a cooling down to -40 °C for *scl*-
160 PHA or -70 °C for *mcl*-PHA at 10 °C.min⁻¹. A second isotherm at -40 °C or -70 °C was kept for 2 min
161 then the sample was heated from -40 to 190 °C or -70 °C to 80 °C at 10 °C.min⁻¹ according to the PHA
162 type. Melting temperatures and melting enthalpies were measured from the first heating ramp for
163 while glass transition temperatures were measured on the second heating ramp and values correspond
164 to the inflection point (Appendice A).

165

166 2.3. Experimental setup of the biodegradation assay

167 A two phase stepwise experiment (Appendix B) was designed in order to evaluate the
168 biodegradability of the polymers under marine conditions, as previously described (Cheng et al.,
169 2022). Briefly, the first step consisted of the formation of a mature biofilm on each PHA groups :
170 PHBHV (from Tianan biological materials, China, 40 µm thickness) was used for the colonization of
171 the *scl*-PHA group including PHB, PHBHV6 and PHBHV11; PHO (produced as described above, 120
172 µm thickness) was used for the colonization for *mcl*-PHA including PHO, PHN and PHNac) together
173 with a positive control (Cellulose filter colonization, CELLU, Whatman 42, thickness 200 µm) and a
174 negative control (Blow Low Density polyethylene colonization, PE, Symphony Environmental
175 Technology, UK, thickness 50µm). Large rectangular pieces of 13.5 cm² of each polymer type
176 mentioned above were incubated for one month (5 August to 6 September 2021) in separate 2.4 L
177 aquarium with continuous circulating seawater (flow rate ranged from 8 to 12 mL.min⁻¹) pumped in
178 the Banyuls bay (NW Mediterranean Sea). Throughout the experiment, seawater temperature (between
179 19 °C and 24 °C) and salinity (38.5 g.L⁻¹) in the aquarium were similar to seawater from the Banyuls
180 bay. Secondly, individual biofilms were detached from two pieces of each PHA or PE or Cellulose
181 colonized films by three cycles of 1 min vortex and 3 min sonication and resuspended in a 40 mL of
182 carbon-minimum medium called “MM” hereafter (Appendix C). Cell numbers were verified by flow
183 cytometry (FACSCanto II flow cytometer, BD Bioscience, San Jose, CA) and adjusted to the exact
184 same concentration 10⁵ cells.mL⁻¹ by dilution of each detached biofilm in MM, in order to ensure

185 comparable inoculum concentration between biodegradation tests. Previous tests using different
186 detached biofilm concentration (from 10^4 to 10^6 cells.mL⁻¹) showed that it was an optimal
187 concentration under our experimental conditions, according to the carrying capacity of the biofilm on
188 plastics (data not shown) and also in accordance to the classical bacterial concentration found in
189 seawater (Pulido-Villena et al., 2012). Three sterile discs of 6 mm² diameter each of each PHA
190 solvent-casted films (PHB, PHBHV6, PHBHV11, PHO, PHN and PHNac, total surface of 60 ± 0.5
191 mm²), PE (total surface of 59 mm²) and Cellulose (total surface of 64 mm²) were then placed in sterile
192 12 mL Exetainer tubes (Exetainer flat bottom 12 mL, Labco, Lampeter, UK) together with 3 mL of the
193 corresponding inoculum previously detached. The tubes were incubated in the dark at $18 \pm 0.25^\circ\text{C}$
194 under agitation at 110 rpm (orbital agitator, Innova® S44i, Eppendorf, Germany) for a 2-month period
195 (called “biotic conditions” hereafter). In addition, similar incubation and sampling procedure were
196 used for abiotic controls, which consisted of triplicate vials containing 3 mL of MM with plastics of
197 the same composition (called “abiotic condition” hereafter). A total of 887 tubes were needed to
198 follow the different parameters detailed below, with triplicates samples taken after 0, 1, 15, 30 and 60
199 days of incubation.

200 2.4. Continuous oxygen measurement

201 During the second step of the experiment, duplicate vials with each plastic type were equipped
202 with an optical fiber luminescent oxygen sensor (SP-PSt5, Presens, Germany) and oxygen
203 concentration was monitored using a small 24-channel reader (SDR SensorDish®, Presens, Germany).
204 Oxygen sensors were placed in the liquid phase to obtain the concentration of dissolved oxygen
205 recorded every hour over 60 days. In the case of *scl*-PHA and cellulose, vials were opened under a
206 sterile laminar flow hood after 30 days to ensure that oxygen was not limiting for bacterial growth and
207 closed again. In this case, oxygen concentration was always maintained at $> 50 \mu\text{mol L}^{-1}$ (20%
208 absolute oxygen), and the re-opening for 10 minutes was enough to return to initial values (around 235
209 $\mu\text{mol L}^{-1}$), as previously described (Cheng et al., 2022). Oxygen consumption was expressed in
210 $\mu\text{mol}(\text{O}_2).\text{mm}^2$. Total surface of the three discs were taken into account: $3 \times (\text{top and bottom: } \pi \times r^2 \text{ and}$
211 $\text{exposed edges: } 2 \times \pi \times r \times h)$.

212 2.5. Heterotrophic Bacterial Production

213 Heterotrophic Bacterial Production (BP) was measured on triplicate samples for each PHA
214 type at 15, 30 and 60 days by ^3H -leucine incorporation into proteins, as previously described (Dussud
215 et al., 2018). Briefly, a soft cell detachment pre-treatment based on three cycle of vortex and
216 sonication was first performed. Then, ^3H -leucine (specific activity of $112 \text{ Ci}.\text{mmol}^{-1}$) was added onto
217 PHA samples (final concentration of $1 \text{ nmol}.\text{L}^{-1}$ after addition of cold leucine). Radioactivity was
218 measured using a Beckman Scintillation Counter (LS 5000CE) after addition of trichloroacetic acid
219 (TCA) 50% and resuspension in a liquid scintillation cocktail (Ultima Gold). An empirical conversion
220 factor of $1.55 \text{ ng C}.\text{pmol}^{-1}$ of incorporated leucine was used to calculate BP (Simon and Azam, 1989).
221 Blanks followed the same protocol but bacterial activity was stopped by the introduction of 50% TCA
222 prior to the addition of the radioactive mix. BP was expressed in $\text{ng}(\text{C}).\text{mm}^2.\text{h}^{-1}$. Total surface of one
223 disc was taken into account: (top and bottom: $\pi \times r^2$ and exposed edges: $2 \times \pi \times r \times h$).

224 2.6. DNA extraction and sequencing.

225 Plastic pieces were sampled at 15, 30 and 60 days and stored at -80°C until DNA extraction.
226 One litre seawater was sampled from the control aquarium, then successively filtered through 3- μm

227 and 0.2- μm pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore), and filters were stored
228 at -80°C . We also sampled the initial biofilm previously detached after the first one-month
229 colonisation step on PHBHV, PHO, cellulose and PE films, which was stored at -80°C after filtration
230 onto 0.2- μm pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore). DNA extractions were
231 realized on all samples using the same phenol-chloroform method, as previously described (Odobel et
232 al., 2021). Primers used for PCR amplification of the 16S V3–V5 region were 515F-Y and 926R
233 (Fuhrman et al., 1989), previously shown as well-suited for marine samples (Parada et al., 2016).
234 Sequencing was performed on Illumina MiSeq by Genoscope (Evry, France), generating 3,060,721
235 paired sequences in the 29 samples. Raw FASTA files were deposited at EBI under the accession
236 number ERP148254. Sequence analysis was processed using the package DADA2 (Version 1.24.0)
237 into R studio software (R Core Team, 2022, version 4.2.2). A standard pipeline was applied with the
238 following parameters: trimLeft= c(19,20), truncLen= c(240,240), maxN=0, maxEE=c(2,2), truncQ=2.
239 The sequences were therefore filtered, dereplicated, denoised by removing sample interference and
240 chimeras before merging. Clusters were assigned with the Silva 128 16S rRNA database (Quast et al.,
241 2013) and clusters that did not belong to Bacteria kingdom were removed as well as chloroplast and
242 mitochondrial sequences. The number of sequences per sample was normalized by rarefaction ($n=$
243 21,324) and a table with 29 samples and 5,053 amplicon sequence variants (ASV) was obtained.

244 2.7. Statistical analysis

245 All graphical representations and statistical analysis were performed on R studio software (R
246 Core Team, 2022, version 4.2.2) using the packages ggplot2 (Wickham, 2016), vegan (Oksanen et al.,
247 2007) and phyloseq (McMurdie and Holmes, 2012) and PRIMER6 (Clarke and Gorley, 2006). Data
248 were compared with Kruskal-Wallis tests followed by post hoc tests at D60 for oxygen consumption
249 and heterotrophic bacterial production. Sequences were analyzed with the phyloseq package. The
250 alpha diversity indexes were calculated and compared with Wilcoxon tests. Differences in microbial
251 community structure among samples were tested by ANOSIM based on Bray-Curtis distances
252 (PRIMER6 software). The ASVs that contributed most to differentiate microbial community structures
253 between *scl*-PHA vs. PE, *scl*-PHA vs. *mcl*-PHA and *mcl*-PHA vs. PE were tested with a similarity
254 percentage analysis (SIMPER, PRIMER6) (Clarke, 1993).

255 3.Results

256 3.1. Physico-chemical characterization of the six tailor-made PHA

257 Three fermentation processes in a bioreactor with *Halomonas sp. SF2003* growing on different
258 substrates resulted in the production of three *scl*-PHA: PHB, PHBHV6 and PHBHV11 (Table 1).
259 *Halomonas sp. SF2003* growing on glucose accumulated a homopolymer of PHB composed at 100%
260 of 3-hydroxybutyrate units (HB) (Table 1) with 4.00 carbons per monomer. A mix of glucose and
261 valeric acid (70/30, % mol) resulted in PHBHV11 production composed of 89% of HB and 11% of
262 HV, leading to an average number of 4.11 carbons per monomer. PHBHV6 was produced using
263 another mix of glucose and valeric acid (50/50, % mol) resulting in a copolymer composed of 94% of
264 HB and 6% of HV with an average number of 4.06 carbons per monomer. Fermentations from
265 *Pseudomonas putida KT2440* from two different fatty acids (octanoic and nonanoic acid) and a β -
266 oxidation pathway inhibitor (acrylic acid) (Jiang et al., 2013) led to three *mcl*-PHA: PHO, mainly
267 composed of 3-hydroxyoctanoate (HO) monomer (89%), then 3-hydroxyhexanoate (HHx) and 3-
268 hydroxydecanoate (HD) (5.5% each) for an average number of 7.78 carbons per monomer, PHN was
269 composed of 3-hydroxynonanoate (HN) monomer units (58%) plus HD (24%), 3-hydroxyheptanoate
270 (HHp) (14%) and HO (4%) units with an average number of 8.92 carbons per monomer and PHNac
271 composed of HN (73%), HHp (23%) and HD (2%) with an average number of 8.47 carbons per
272 monomer.

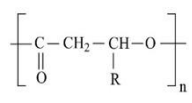
273 Thermal properties of all PHA were characterized by DSC (Table 1, Appendix A). *Scl*-PHA
274 displayed glass transition temperatures between -7°C and 4°C . *Scl*-PHA with HV units (PHBHV6 and
275 PHBHV11) showed slightly lower peaks of melting temperatures (171 for PHBHV6 and 172°C for
276 PHBHV11) compared to PHB (177°C). Melting enthalpies also differed according to the chemical
277 composition. Higher melting enthalpy is observed for PHB ($\Delta H_m = 89 \text{ J.g}^{-1}$) then followed by
278 PHBHV6 ($\Delta H_m = 60 \text{ J.g}^{-1}$) and finally by PHBHV11 ($\Delta H_m = 30 \text{ J.g}^{-1}$) due to the presence of HV units
279 which partially limit the chain crystallisation. Number average molecular mass could not be measured
280 for PHB due to solubility difficulties. PHBHV6 and PHBHV11 displayed \overline{M}_n of 340 000 and 325 000
281 g.mol^{-1} with a dispersity index (\mathfrak{D}) of 2.8 and 2.9, respectively. Among *scl*-PHA, contact angles with

282 distilled water revealed that PHB is the least hydrophobic polymer with a contact angle of $64 \pm 1.6^\circ$,
 283 followed by PHBHV11 ($74 \pm 1^\circ$) and PHBHV6 ($76 \pm 2^\circ$). On the other hand, *mcl*-PHA displayed
 284 lower glass transition temperatures (PHO: -36°C , PHN: -40°C and PHNac: -39°C) and lower
 285 melting temperatures (PHO: 57°C , PHN: 49°C and PHNac: 52°C) than all *scl*-PHA. Melting
 286 enthalpies is also far lower than *scl*-PHA with a melting enthalpy of $20 \text{ J}\cdot\text{g}^{-1}$ for PHO and PHN and a
 287 lower one of $13 \text{ J}\cdot\text{g}^{-1}$ for PHNac. \overline{M}_n of *mcl*-PHA ranged from 60 000 to 84 000 $\text{g}\cdot\text{mol}^{-1}$ with a \mathfrak{D}
 288 ranging from 2.2 to 2.7. High hydrophobicity is displayed by *mcl*-PHA, especially for PHN ($90 \pm 2^\circ$)
 289 then for PHO and PHNac ($82 \pm 2^\circ$ and $80 \pm 1^\circ$, respectively). Overall, physico-chemical properties of
 290 the PHA produced differed greatly according to the type of PHA (*scl*- or *mcl*-) while slight but
 291 noticeable differences were found within both PHA types.

292

293 **Table 1:** Composition of the 6 tailor-made PHA and their associated thermal characteristics, average
 294 molecular mass and contact angle.

295

	Chemical composition	Average number of carbon per monomer	\overline{M}_n ($\text{g}\cdot\text{mol}^{-1}$)	\mathfrak{D}	T_g ($^\circ\text{C}$)	T_m ($^\circ\text{C}$)	ΔH_m ($\text{J}\cdot\text{g}^{-1}$)	Contact angle ($^\circ$)
PHB	100% HB	$C_{4.00}$	-	-	4	177	89	64 ± 2
PHBHV6	94% HB 6% HV	$C_{4.06}$	340 000	2.8	-7	171	60	76 ± 2
PHBHV11	89% HB 11% HV	$C_{4.11}$	325 000	2.9	-7	172	30	74 ± 1
PHO	5.5% HX 89% HO 5.5% HD	$C_{7.78}$	84 000	2.2	-36	57	20	82 ± 2
PHN	14% HHp 4% HO 58.1% HN 24% HD	$C_{8.92}$	60 000	2.7	-40	49	20	90 ± 2
PHNac	23% HHp 74% HN 2% HD	$C_{8.47}$	70 000	2.2	-39	52	13	80 ± 1

296

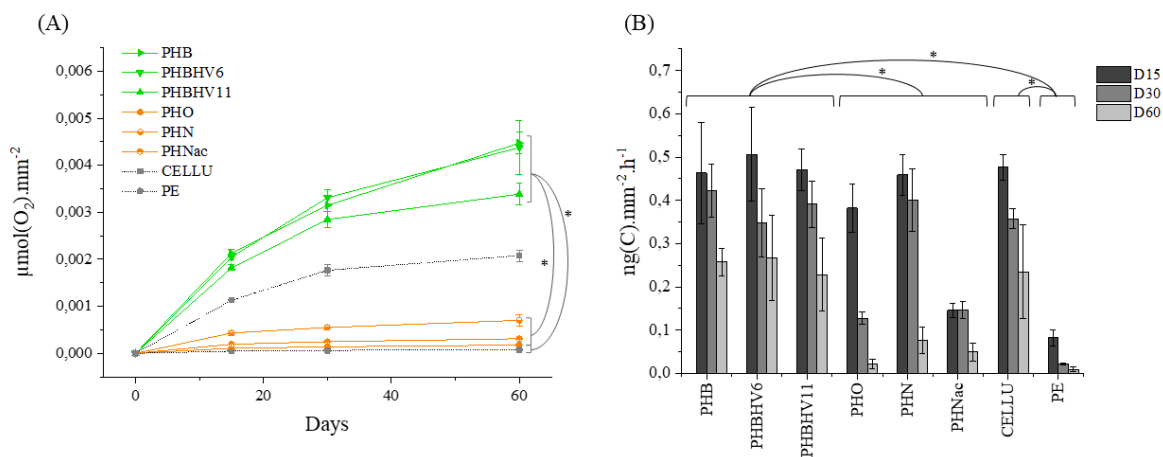
297

298 3.2. Biodegradation activities

299 Several parameters were used to evaluate the biodegradability of the various PHA. Firstly,
300 abiotic controls (PHA, CELLU or PE) did not show signs of contamination and chemical oxygen
301 demand was negligible in our conditions.

302 Secondly, continuous oxygen consumption (Presens sensors) by microorganisms with PHA as
303 sole carbon and energy source showed a clear distinction between *scl*- and *mcl*-PHA. During the first
304 15 days, oxygen consumption rapidly increased and tended to reach a plateau from 15 to 60 days for
305 the *mcl*-PHA while it kept increasing to a greater extent for *scl*-PHA (Fig. 1A). At day 60, PHB (mean
306 = $4.47 \pm 0.23 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$), PHBHV6 (mean = $4.37 \pm 0.57 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$), and
307 PHBHV11 ($3.39 \pm 0.23 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$) presented a much higher oxygen consumption than PHO
308 ($0.18 \pm 0.02 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$), PHN ($0.70 \pm 0.11 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$), and PHNac ($0.31 \pm$
309 $0.02 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$). Significant difference was found between *scl*- and *mcl*-PHA
310 biodegradation ($p < 0.05$), as well as with between *scl*- and the PE control ($0.72 \pm 0.09 \times 10^{-4}$
311 $\mu\text{mol}(\text{O}_2).\text{mm}^{-2}$, $p < 0.05$). No significant difference was found between the oxygen consumption on *scl*-
312 PHA compared to Cellulose ($2.08 \pm 0.12 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$, $p > 0.05$). Likewise, a kinetic
313 comparison between within *scl*-PHA or within *mcl*-PHA did not show any statistical differences.
314 Among *mcl*-PHA, small but noticeable oxygen consumption was observed for PHN, while PHO and
315 PHNac had a similar trend to the PE negative control.

316 Thirdly, cell incorporation of ^3H -leucine into proteins showed similar trends, with significantly
317 higher heterotrophic activities for *scl*-PHA as compared to *mcl*-PHA. Within the first 15 days of
318 incubation in minimum medium, the activities of the biofilms were high and then decreased until day
319 60 for all the PHA (Fig. 1B). At day 60, maximum activities were found for the positive control
320 cellulose ($2.35 \pm 1.08 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$) and *scl*-PHA, including PHBHV6 ($2.67 \pm 0.97 \times 10^{-1}$
321 $\text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$), PHB ($2.58 \pm 0.31 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$) and PHBHV11 ($2.28 \pm 0.85 \times 10^{-1}$
322 $\text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$). Much lower bacterial activities were observed for the negative control PE ($0.95 \pm$
323 $0.58 \times 10^{-2} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$) and *mcl*-PHA, including PHN ($0.77 \pm 0.30 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$), PHNac
324 ($0.49 \pm 0.20 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$), PHO ($0.21 \pm 0.10 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$), that significantly
325 differed from cellulose ($p < 0.05$).



327

328 **Figure 1:** Cumulative oxygen consumption (A) and bacterial heterotrophic production (B) on the
 329 different polymers (green: PHB, PHBV6, PHBV11; orange: PHO, PHN and PHNac and grey:
 330 CELLU and PE) in minimum media for 15, 30, 60 and 90 days. Errors bars indicate standard
 331 deviation. * indicates significant difference by Kruskal-Wallis test on day 60 (n = 24).

332

333 [3.3. Bacterial diversity](#)

334 During the 60-days of experiment, no significant change in alpha-diversity was observed in all
 335 polymer types over time, including all the measured diversity indexes (Chao1 richness, Pielou
 336 evenness, Shannon and Simpson diversity) ($p > 0.05$) (Table 2). However, significant differences were
 337 found between the polymer groups, including cellulose and *scl*-PHA (PHB, PHBV6 and
 338 PHBV11), as compared to another group including PE and *mcl*-PHA (PHO, PHN and PHNac) ($p <$
 339 0.05). Lower Chao1 richness and Shannon diversity were found for *scl*-PHA (244.4 ± 8.8 and $3.3 \pm$
 340 0.3 ; n = 9, respectively) as compared to *mcl*-PHA (606.8 ± 19.4 and 4.4 ± 0.4 ; n = 9, respectively) ($p =$
 341 1.6×10^{-4}). Higher diversity on the free-living bacteria and on the initial inocula for each polymer type
 342 was also observed (Table 2).

343

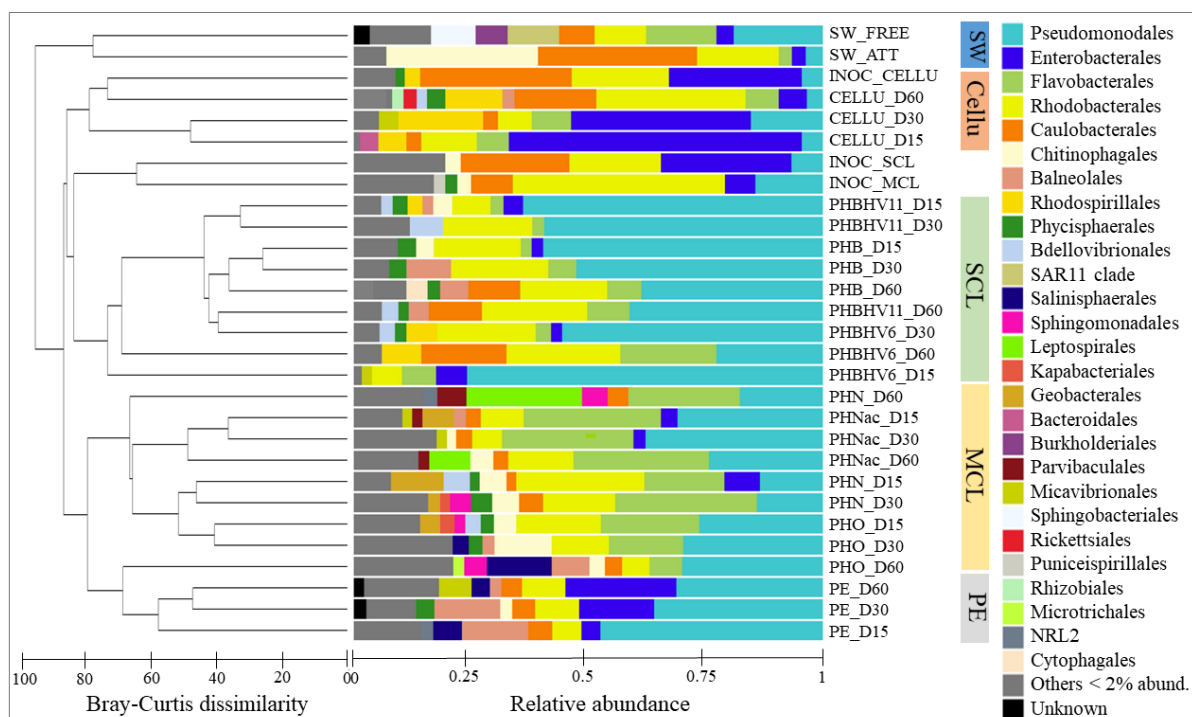
344 Table 2: Total number of ASV per sample together with *Chao1* richness, Pielou evenness and Shannon,
 345 and Simpson diversity indexes. SW: seawater, INOC: microbial inoculum from each polymer types
 346 after one month of colonization, D: incubation time in days.

Sample	Total ASV	Chao1	Pielou	Shannon	Simpson
SW-FREE	939	1313	0.716	4.9	57.8
SW-ATT	497	541	0.644	4	18.3
INOC_CELLU	707	889	0.671	4.4	25.5
CELLU_D60	207	226	0.656	3.5	15.5
CELLU_D30	288	322	0.636	3.6	12.7
CELLU_D15	128	129.	0.556	2.7	5.8
INOC_SCL	651	656.	0.818	5.3	75.4
INOC_MCL	1276	1425	0.755	5.4	42.9
PHBHV11_D15	307	331	0.559	3.2	8.2
PHBHV11_D30	166	171	0.548	2.8	6.3
PHB_D15	313	343	0.574	3.3	11.4
PHB_D30	243	264	0.564	3.1	11.4
PHB_D60	213	246	0.653	3.5	18
PHBHV11_D60	266	294	0.591	3.3	10.9
PHBHV6_D30	212	237	0.672	3.6	14.8
PHBHV6_D60	156	163	0.733	3.7	27
PHBHV6_D15	147	148	0.561	2.8	8
PHN_D60	423	528	0.678	4.1	23.5
PHNac_D15	435	470	0.691	4.2	23.8
PHNac_D30	517	560	0.640	4	14.5
PHNac_D60	403	472	0.650	3.9	20.7
PHN_D15	434	498	0.692	4.2	25.4
PHN_D30	290	302	0.776	4.4	45.3
PHO_D15	689	767	0.719	4.7	48.7
PHO_D30	753	879.	0.740	4.9	58.4
PHO_D60	822	983	0.775	5.2	74.7
PE_D60	711	828	0.701	4.6	31.3
PE_D30	616	676	0.747	4.8	65.5
PE_D15	547	614	0.793	5	82.7

348

349 Beta-diversity analysis showed four distinct groups between bacterial communities living on
350 cellulose, *scl*-PHA (PHB, PHBHV6 and PHBHV11), *mcl*-PHA (PHO, PHN and PHNac), PE and in
351 seawater (Fig. 2). Interestingly, inoculum grown on cellulose before the experiment grouped with the
352 bacterial communities living on cellulose as sole carbon source. In contrast, this was not the case for
353 *scl*-PHA and *mcl*-PHA inocula, that changed when incubated with the different polymer types.
354 ANOSIM analysis showed significant differences between *scl*-PHA and *mcl*-PHA samples ($R = 0.964$,

355 $p < 0.05$). Within these groups, no clear distinction could be made between samples, except for
 356 PHBHV6 (day 15 and 60) for *scl*-PHA and PHO-D60 for *mcl*-PHA.

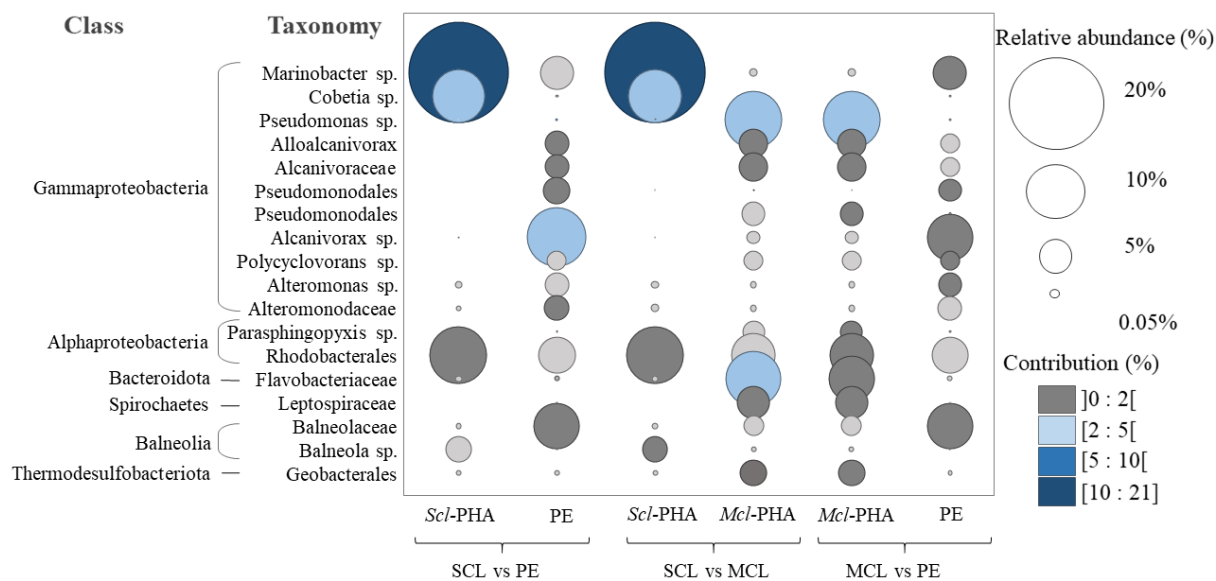


357
 358 **Figure 2:** Comparison of community structures and taxonomic abundances of bacteria in seawater
 359 (SW), on the microbial inoculum from each polymer types after one month of colonization (INOC)
 360 and bacteria on polymers (PHB, PHBHV6, PHBHV11, PHO, PHN and PHNac) according to time (in
 361 days, D), by UPGMA dendrogram based on Bray-Curtis dissimilarities between sequencing profiles
 362 (left) and by cumulative bar charts comparing relative order abundances (right).

363 Taxonomic composition confirmed the niche-partitioning between the bacterial communities
 364 living in seawater compared to the plastisphere of the different polymer types. Free-living bacteria
 365 were composed of Pseudomonadales (19%), Flavobacteriales (15%), Rhodobacterales (11%), SAR11
 366 clade (11%), Sphingobacteriales (9%) and Caulobacterales (8%), while organic particle-attached
 367 bacteria were dominated by Caulobacterales (34%), Chitinophagales (32%) and Rhodobacterales
 368 (17%). The inoculum pre-grown on cellulose in seawater was mainly composed of Caulobacterales
 369 (32% for INOC_CELLU), Rhodobacterales (21% for INOC_CELLU), Enterobacterales (28% for
 370 INOC_CELLU) and to a lesser extent of Pseudomonadales (5% for INOC_CELLU). The same groups
 371 were also found for the inocula pre-grown on *scl*-PHA and *mcl*-PHA but with different proportions,

372 including Caulobacterales (23% for INOC_SCL and 9% for INOC_MCL), Rhodobacterales (20% for
373 INOC_SCL and 45% for INOC_MCL), Enterobacterales (28% for INOC_SCL and 7% for
374 INOC_MCL) and Pseudomonadales (5% for INOC_CELLU, 7% for INOC_SCL and 14% for
375 INOC_MCL). After inoculation with the different polymer types as sole carbon source, the proportion
376 of different taxa was different between the different polymer types. Pseudomonadales were high on
377 *scl*-PHA ($52 \pm 15\%$, n=9), *mcl*-PHA ($25 \pm 2\%$, n=9), PE samples ($28 \pm 7\%$, n=3) and cellulose ($8 \pm$
378 5% , n=3) together with Rhodobacterales (mean = $18 \pm 6\%$, $13 \pm 7\%$, $8 \pm 1\%$, $17 \pm 10\%$, n=9 for *scl*-
379 PHA, *mcl*-PHA, PE and cellulose, respectively). *Mcl*-PHA exhibited high proportions of
380 Flavobacteriales (mean = $22 \pm 7\%$, n = 9) compared to other polymers (between 0% on PE and 10%
381 on others polymer). The main taxa found on cellulose was Enterobacterales (mean = $35 \pm 23\%$, n = 3),
382 especially on CELLU_J15 and CELLU_J30 (Fig. 2).

383 SIMPER analysis on the Bray Curtis dissimilarity index highlighted two species contributing
384 to the differences between *scl*-PHA and PE. First, ASV affiliated to *Marinobacter sp.* showed a high
385 relative abundance in *scl*-PHA (more than 37%) and a high contribution (20%) (Fig. 3) compared to
386 PE. *Cobetia sp.* is the second most specific species found on *scl*-PHA in a lesser abundance (10%) and
387 contributes to 6% on *scl*-PHA. Those two species that display a clear distinction between *scl*-PHA and
388 PE microbial communities also contributed to the differences found between *scl*-PHA and *mcl*-PHA.
389 Indeed, *Marinobacter sp.* and *Cobetia sp.* were poorly represented on *mcl*-PHA, while a major relative
390 abundance and contribution were found for *Pseudomonas sp.* (12% and 7% for relative abundance and
391 contribution, respectively) and Flavobacteriaceae (11% and 6% for relative abundance and
392 contribution, respectively) for this polymer type. Additionally, *Pseudomonas sp.* and
393 Flavobacteriaceae were poorly represented on *scl*-PHA, as well as on PE. Bacterial communities
394 observed on *mcl*-PHA and PE seemed richer and more scattered. With the exception of
395 Rhodobacterales that were abundant on all polymer types, taxa belonging to *Alcanivorax sp.* (13% and
396 8% of relative abundance and contribution, respectively), Balneolaceae, Pseudomonadales,
397 Alloanivorax, Alcanivoraceae and Alteromonadaceae found on PE were poorly abundant on *scl*-
398 PHA. Taxa belonging to Alloanivorax and Alcanivoraceae were also present on *mcl*-PHA but
399 presented low contribution (2%) when compared to PE.



400

401

402 **Figure 3:** Bubble plot showing the relative abundance and the taxonomy of the majority ASV
 403 contributing to 50% of the dissimilarity between *scl*-PHA vs. *mcl*-PHA, *scl*-PHA vs. PE and *mcl*-PHA
 404 vs. PE. Points are sized according to the relative abundance and colored by their contribution to
 405 dissimilarity.

406 4. Discussion

407 4.1. Different physico-chemical characteristics of the six tailor-made PHA

408 The first step of this study was to produce three *scl*-PHA and three *mcl*-PHA with various
 409 intrinsic properties in order to estimate the influence of the physico-chemical characteristics in the
 410 PHA biodegradation in the marine environment. The bacterial strain *Halomonas sp.* SF2003 has been
 411 used for the production of *scl*-PHA using glucose and/or valeric acid to generate PHB, PHBV6 and
 412 PHBV11. Surprisingly, an increase of valeric acid in the substrate feeding led to a lower HV content
 413 in PHBV6. Valeric acid was added to the medium in the form of a mix of valeric acid and glucose.
 414 Since it was continuously dropped at a slow rate (4 mL.min⁻¹), it led to the permanent presence of low
 415 valeric acid concentration in the bioreactor but also glucose concentration. *Halomonas sp.* SF2003
 416 cells seem, in this particular conditions (on two permanently available substrates), metabolize glucose
 417 for HB accumulation at the expense of valeric acid and therefore HV incorporation in the polymer.
 418 The use of different substrates induced different chemical compositions that affect thermal properties.
 419 HV incorporation tended to slightly lower the glass transition temperatures changed from -7 to 4 °C

420 with 11 and 6% of HV proportions. Melting temperatures dropped from 177 °C to 172 °C and 171 °C
421 with 11% and 6% of HV incorporation, respectively. Overall, DSC analysis showed common features
422 found in other *scl*-PHA produced and characterized in the literature (Koller et al., 2010; Lemechko et
423 al., 2019; Mozejko-Ciesielska and Kiewisz, 2016). The melting enthalpy, comparable to the PHA
424 crystallinity, was a parameter influenced by the HV incorporation due to a higher steric hindrance
425 generated by the HV units compared to that of HB units. It tended to decrease as much as the HV
426 content increased to reach 30 J.g⁻¹ with 11% of HV unit. The modulation of HV unit proportion, even
427 at low incorporation, is a promising way to modify the PHA properties, for instance to bring softness
428 and elasticity to the homopolymer PHB, which can show some difficult features to process due to its
429 high crystallinity and a melting temperature close to its degradation temperature (Pachekoski et al.,
430 2013). Regarding *mcl*-PHA, different chain lengths and/or monomer proportions have been achieved,
431 thus impacting to a certain extent the intrinsic properties of each of these *mcl*-PHA. PHO was mainly
432 composed of HO (89%), PHN was mainly composed of HN (58%) and the addition of acrylic acid
433 improved the HN content in PHNac (74%). Improving the HN content in PHNac seemed to mainly
434 modify the crystallinity, since the melting enthalpy drops from 20 to 13 J.g⁻¹ in this latter polymer.
435 Generally, thermal properties between *mcl*-PHA slightly differ and are in accordance with PHA
436 composed of these monomers (Abe et al., 2012; Mozejko-Ciesielska and Kiewisz, 2016). Tailor-made
437 production with different strains and carbon sources allowed to produce PHA displaying clear and
438 significant distinctions between *scl*- and *mcl*-PHA, including thermal properties, molecular weights or
439 hydrophobicity.

440

441 4.2. Various biodegradation activities on the different polymer types

442 The main originality of our study is to present pioneer results of microbial biodegradation
443 activities of the various tailor-made PHA under natural marine conditions. Particular attention has
444 been made here to produce PHA samples through the same process and of the same shape and size.
445 Special care was also taken to mimic the polymer biodegradation capabilities of natural mature biofilm
446 growing on plastics, by using pre-colonized biofilms on each *scl*- and *mcl*-PHA as test inoculum (or
447 biofilm growing on PE and cellulose for controls), as previously described (Cheng et al., 2022).

448 Previous studies underlined the lack of environmental representability of standard test methodologies,
449 which was due in particular to the inadequate test inoculum (Harrison et al., 2018; Napper and
450 Thompson, 2020; Paul-Pont et al., 2023). In particular, the use of seawater as test inoculum is not
451 representative of the biofilm growing on plastic, since the two communities were shown to clearly
452 differ in term of biodiversity and functions (Bryant et al., 2016; Zettler et al., 2013). Moreover,
453 mature biofilm formed on conventional plastic (such as PE) were shown to be different from
454 biodegradable plastics (such as PHA or cellulose) (Odobel et al. 2021). This is the reason why the first
455 step consisted of the formation of a mature biofilm on each PHA groups (*scl*- and *mcl*-PHA), as well
456 as on PE and cellulose controls. In addition, a minimum medium with no carbon source was used to
457 avoid false positive signals, together with the addition of nutrient according to Redfield N:P ratio
458 classically used in marine biodegradation tests (16:1) (Van Wambeke et al., 2009). Most of the
459 evidence for PHA biodegradability in marine environment focused previously on weight loss (Deroiné
460 et al., 2015, 2014; López-Ibáñez and Beiras, 2022; Volova et al., 2011). Weight loss provides a proof
461 of the plastic disintegration only, which may or may not be associated to the complete mineralization
462 by bacteria (Haider et al., 2019). There is a consensus in using the last mineralization step as a relevant
463 proof of plastic biodegradability, either estimated by O₂ uptake or CO₂ release (Jacquin et al. 2019).
464 Here, we measured the O₂ uptake directly on the aqueous phase by using the ‘plastic-free’ Presens®
465 optical dissolved oxygen sensors, which have been proven to give similar response and with less
466 abiotic losses compared to other commercially available manometric test systems, such as the
467 Oxitop® device (Brown et al. 2018). It was particularly well suited for the large number of replicate
468 samples tested in this study, and allowed the use of 12mL Exetainer tubes with perfect sealing that
469 fitted in only one incubator for better reproducibility and with strict thermal regulation ($\pm 0.25^{\circ}\text{C}$) that
470 reduced variation in O₂ values. With PHA being the sole carbon source in our biodegradation tests, the
471 trend of oxygen consumption and microbial activities on *scl*-PHA clearly demonstrated their
472 biodegradability in seawater, thus confirming previous observations by using other techniques
473 (Deroiné et al., 2015, 2014; Volant et al., 2022).

474 Under laboratory conditions and by using pure bacterial culture, PHA biodegradation
475 processes were depicted to be the result of specialized extracellular enzymes called PHA

476 depolymerases (Leathers et al., 2000; Mukai et al., 1993). The enzymes are capable of hydrolysing
477 PHA chains into smaller water-soluble compounds (< 600 Da) that can cross the membranes for
478 further bacterial degradation and assimilation (Azam and Malfatti, 2007). In our study, respiration
479 associated to the *scl*-PHA assimilation by bacteria resulted in a regular increase in oxygen
480 consumption during the 60 days of biodegradation tests. The respiration rates were higher than with
481 the cellulose positive control in the same experimental conditions, whereas it was almost undetectable
482 on PE negative control. Bacterial heterotrophic activity (³H-Leucine incorporation) on *scl*-PHA as sole
483 carbon source showed the same trend, with significantly higher activity on *scl*-PHA than for *mcl*-PHA
484 and PE. Both oxygen consumption and bacterial heterotrophic activities were high during the first 15
485 days of tests (even for PE films to a lesser extent), likely due to the organic matter that was detached
486 together with the pre-colonized biofilm or due to mortality, thus rendering this period of the
487 biodegradation tests less adequate for biodegradation measurement under our conditions. A similar
488 difference in bacterial heterotrophic production between PHBHV and PE films was previously found
489 during long-term colonization and biodegradation (Dussud et al., 2018; Odobel et al., 2021). Within
490 the *scl*-PHA group, we observed slight but significantly higher oxygen consumption on PHB and
491 PHBHV6 compared to PHBHV11 after 60 days. Such a difference was not found for bacterial
492 heterotrophic activities, rendering the difference in biodegradation within the *scl*-PHA less robust.
493 Contrasting results found in the literature confirmed the possible but not consistent difference in
494 biodegradation rates for these two polymers. A hypothesis of better biodegradation abilities of the
495 PHBHV copolymer was linked to an increase of amorphous regions which are more susceptible to
496 enzymatic attack compared to the homopolymer PHB (Meereboer et al., 2020; Numata et al., 2008).
497 Other studies of *in vitro* enzymatic degradation showed the opposite, with better degradation
498 capacities on PHB compared to PHBHV (Mukai et al., 1993). Slight differences in terms of
499 biodegradation between *scl*-PHA are therefore difficult to explain since biodegradation is a
500 combination of physical, chemical and biological factors (Dilkes-Hoffman et al., 2019). As a
501 consequence, we conclude that the intrinsic differences within the *scl*-PHA properties
502 (hydrophobicity, crystallinity, molecular weight) were not sufficient to induce a difference in
503 biodegradation activities in our marine experimental conditions.

504 In the opposite, signs of biodegradation were very low or almost undetectable for the tested
505 *mcl*-PHA types. By comparison to *scl*-PHA, very few studies tested the biodegradability of *mcl*-PHA
506 in marine ecosystems, probably because no *mcl*-PHA are commercially available (Lott et al., 2021;
507 Suzuki et al., 2021). The tailor-made *mcl*-PHA produced in this study showed clear distinct chemical
508 differences between PHO, PHN and PHNac. Although PHN showed a slightly higher oxygen
509 consumption and bacterial heterotrophic activities after 60 days than PHO and PHNac, no statistical
510 difference was shown. It is to be noted that oxygen consumption and bacterial heterotrophic activities
511 of the *mcl*-PHA group were similar to the negative control PE, which was a sign of very low or no
512 biodegradability in our marine experimental conditions. We are aware that the 2-month timing of tests
513 was probably not sufficient and we propose to perform further studies with a longer test period before
514 giving a firm conclusion of the absence of biodegradability (in a reasonable period of time) for the
515 *mcl*-PHA.

516 Interestingly, our study offers a large set of analysis to compare the physico-chemical
517 characteristics of *scl*- and *mcl*-PHA and assess their impact on PHA biodegradation. Although
518 polymers with low number average molecular weight, low crystallinity and low hydrophobicity are
519 expected to show better sign of biodegradation (Kumar et al., 2020), it does not seem to fully explain
520 the difference found in *scl*- and *mcl*-PHA biodegradability with natural inoculum. Indeed, *scl*-PHA
521 produced in this studies were more crystalline with higher number average molecular weight than the
522 *mcl*-PHA but they still showed far greater biodegradation abilities. Then, the differences in physico-
523 chemical characteristics between *scl*- and *mcl*-PHA might not be sufficient to explain the difference
524 observed on biodegradation. As mentioned in previous studies of PHA biodegradation in seawater
525 (Deroiné et al., 2015), no significant changes were observed in molecular weight at the end of the
526 experiment, thus confirming a enzymatic process of degradation that resulted in surface erosion rather
527 than bulk erosion (Appendix D). We hypothesize that biodegradation might also result from the
528 specificity of the extracellular PHA depolymerase to the *scl*- or to the *mcl*-PHA. Indeed, it has been
529 previously shown that the catalytic domain activity differed between *scl*- and *mcl*-PHA depolymerase,
530 rendering the *mcl*-PHA depolymerase ineffective on *scl*-PHA, and inversely (Kim et al., 2000).
531 Moreover, the carbon chain length of *mcl*-PHA (which present a higher molecular mobility compared

532 to those of *scl*-PHA) could inhibit enzymatic degradation by impeding the catalytic domain with
533 longer side chain length and steric hindrance interferences (Numata et al., 2009). Finally, *mcl*-PHA
534 depolymerases are less abundant than *scl*-PHA depolymerases in several type of environments
535 including the marine environment (Viljakainen and Hug, 2021). These results suggest that the type of
536 PHA mainly influences the biodegradation rate.

537

538 4.3. Dissimilar microbial community associated to the various polymer types

539 Biodegradation is a complex process involving intrinsic (relative to the polymer) but also
540 extrinsic factors (relative to the environment). In this study, we decided to keep the same temperature,
541 mixing, light and nutrients constant to focus on the impact of bacterial diversity as a key factor in
542 plastic biodegradation. First, we paid specific attention to performing the biodegradation tests with
543 realistic biofilm living in the natural marine environment. While there is no consensus today for the
544 preparation of the microbial inocula in the ISO or ASTM standards for polymer biodegradability tests,
545 convergent views indicated that complex natural marine inoculum made of biofilm growing on the
546 corresponding plastics under natural conditions are recommended (Cheng et al., 2022). A
547 colonisation phase for a minimum of one month in natural seawater has been shown to be a pre-
548 requisite to mimic a mature biofilm in seawater (Jacquin et al., 2019; Odobel et al., 2021), as has been
549 done in this study. Bray-Curtis similarity showed that the biofilms growing during one month in
550 natural seawater were similar in *scl*-PHA and *mcl*-PHA, but different from cellulose or PE films. As
551 previously described in other studies, free-living and particle-attached bacteria living in the seawater
552 presented very different communities compared to the plastisphere of the mature biofilms (Dussud et
553 al., 2018; Wright et al., 2020), thus rendering the use of seawater as inoculum for biodegradation tests
554 irrelevant. We emphasize the value of using inoculum made of pre-formed mature biofilm as an
555 important methodological step forward for biodegradation tests, as previously described (Cheng et al.,
556 2022; Jacquin et al., 2019).

557 The transfer of pre-formed biofilm from natural seawater to minimum medium resulted in
558 bacterial community changes for *scl*-PHA and *mcl*-PHA, but not for cellulose that remained stable

559 during the entire 60-day incubation. Following the evolution of the bacterial community changes
560 during the biodegradation tests has been recommended by previous studies (Jacquin et al., 2019;
561 Kowalczyk et al., 2015), but this recommendation has been poorly followed thereafter. Changes in
562 bacterial diversity may be used as a signal of the lack of representability of the biodegradation tests to
563 mimic the natural environment. By following the bacterial diversity for all the tested plastics, we
564 showed that the alpha-diversity remained stable during the course of the second step of the experiment
565 for all plastic types, which is a prerequisite for the biodegradability tests in natural conditions (Jacquin
566 et al., 2019).

567 The study of the bacterial communities also permitted to describe the potential of some ASVs
568 to be involved in the biodegradation of the *scl*-PHA. SIMPER analysis on Bray-Curtis 16S rRNA
569 dissimilarities showed the importance of *Marinobacter* sp. and *Cobetia* sp. in explaining the
570 difference between the *scl*-PHA and the negative control PE. *Marinobacter* sp. has been previously
571 shown to present abilities to degrade PHB and PHBHV (Kasuya et al., 2000; Martínez-Tobón et al.,
572 2018). These authors demonstrated PHB and PHBHV depolymerase activities of isolated
573 *Marinobacter* strains, and identified the *scl*-PHA depolymerase PhaZ gene. *Cobetia* sp. has never been
574 observed as PHA-degraders, but it is a well-known producer of PHA (Christensen et al., 2021; Moriya
575 et al., 2020). Further studies are needed to evaluate its potential to perform both the production and the
576 degradation of *scl*-PHA using exoenzymes, as it has been shown for other species (Martínez-Tobón et
577 al., 2018; Nygaard et al., 2021). These two strains were much less abundant in PE but also in *mcl*-
578 PHA, thus suggesting a selection in *scl*-PHA associated to its biodegradation under marine conditions.
579 Some specific species were detected in *mcl*-PHA that presented low abundance in PE. This is
580 particularly the case for *Pseudomonas* sp., which were previously shown as very effective producers
581 of *mcl*-PHA (Prieto et al., 2016), with the ability to also produce extracellular *mcl*-PHA depolymerase
582 (Schirmer et al., 1993; Schirmer and Jendrossek, 1994; Young et al., 2005). *Pseudomonas* sp. was also
583 very low in abundance in *scl*-PHA, confirming the selection of different species depending on the *scl*-
584 PHA vs. *mcl*-PHA groups. Further long-term studies will be needed to evaluate if the *Pseudomonas*
585 sp. selected on *mcl*-PHA may be involved in their biodegradation. This first study on *mcl*-PHA opens

586 new routes for further studies to better understand the bacterial diversity involved in their
587 biodegradation in the marine environment.

588 Conclusion

589 PHA are generally cited as one solution among others to replace conventional plastics, that
590 would be both bio-based and biodegradable. Most of the studies so far have proven the rapid
591 biodegradability of *scl*-PHA that are already commercially available, but very few of them
592 investigated the fate of *mcl*-PHA in the environment. To our knowledge, this is the first study
593 comparing the biodegradation of *scl*- and *mcl*-PHA in the marine environment. One strength of our
594 work was to produce six tailor-made PHA with different physico-chemical characteristics, in order to
595 estimate their biodegradation and identify their associated bacterial community. The physico-chemical
596 properties of the PHA studied might not be sufficiently different to have an impact of these
597 characteristics on biodegradation signals within a PHA type. However, this study showed that the
598 chemical nature of the polymer (*short*- vs. *medium-chain* length PHA) together with the diversity of
599 microorganisms living on the plastic films (and probably the associated enzymes, i.e. PHA
600 depolymerase) were the main drivers of the PHA biodegradability in the marine environment. These
601 results are of importance for further application of PHA with different rates of biodegradation for
602 commercial purpose, such as the production of fishing nets, buoys or cosmetic products that
603 potentially end their life in the marine environment (Paul-Pont et al., 2023). This study also showed
604 that *mcl*-PHA biodegradation takes longer than *scl*-PHA, which could suggest the use of this PHA
605 group for longer-lifetime products. Further biodegradation tests with longer period of time (more than
606 2 months) are needed to better explore the biodegradation of the more recalcitrant *mcl*-PHA, and we
607 believe that this study opens new routes for a better understanding of *scl*-PHA and *mcl*-PHA
608 biodegradation in the marine environment.

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610

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622 [Conflict of interest:](#)

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625

626 [Author Contributions \(CRediT taxonomy\)](#)

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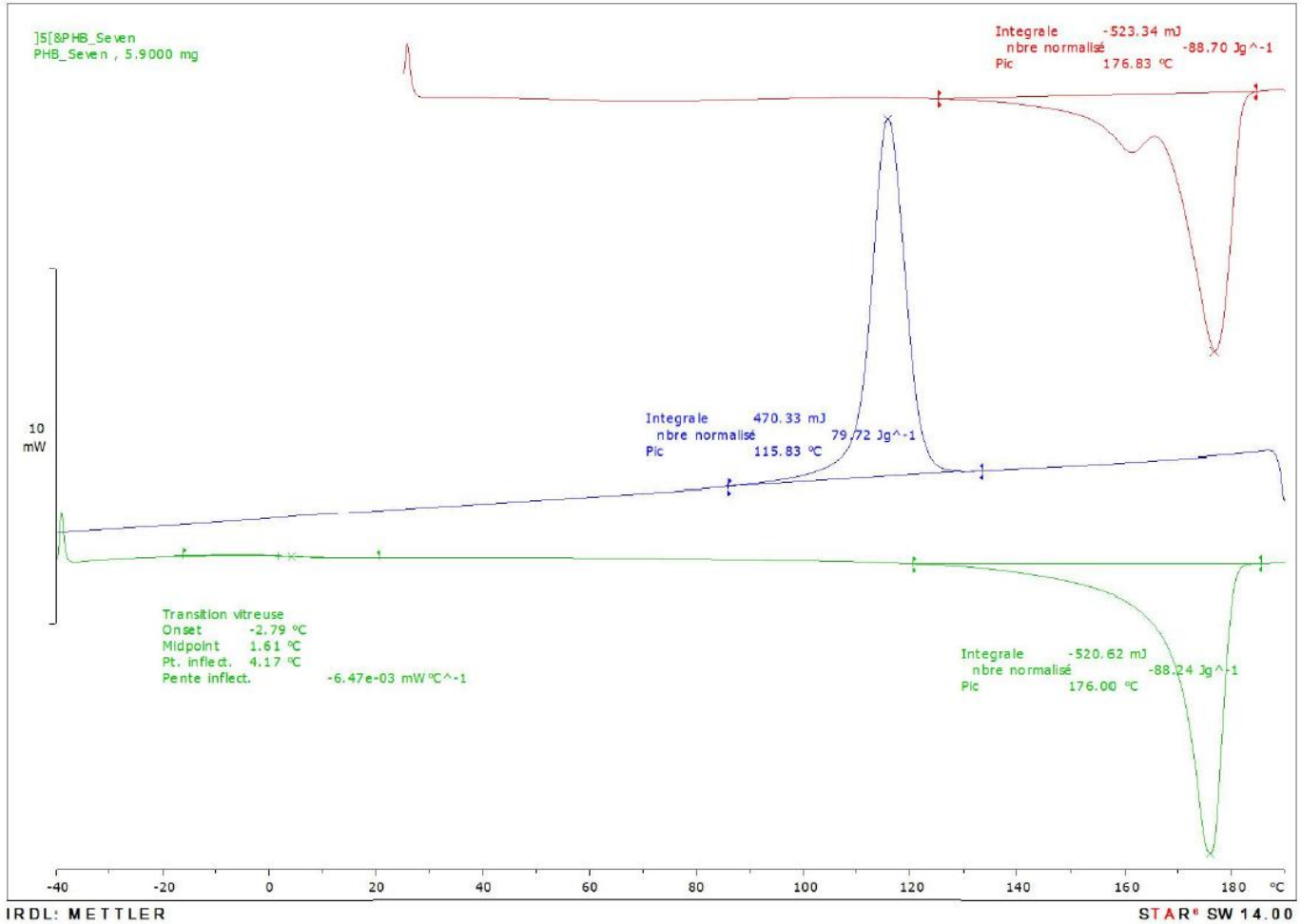
861 Appendices

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863 Appendix A: DSC curves of the 6 PHA solvent-casted films

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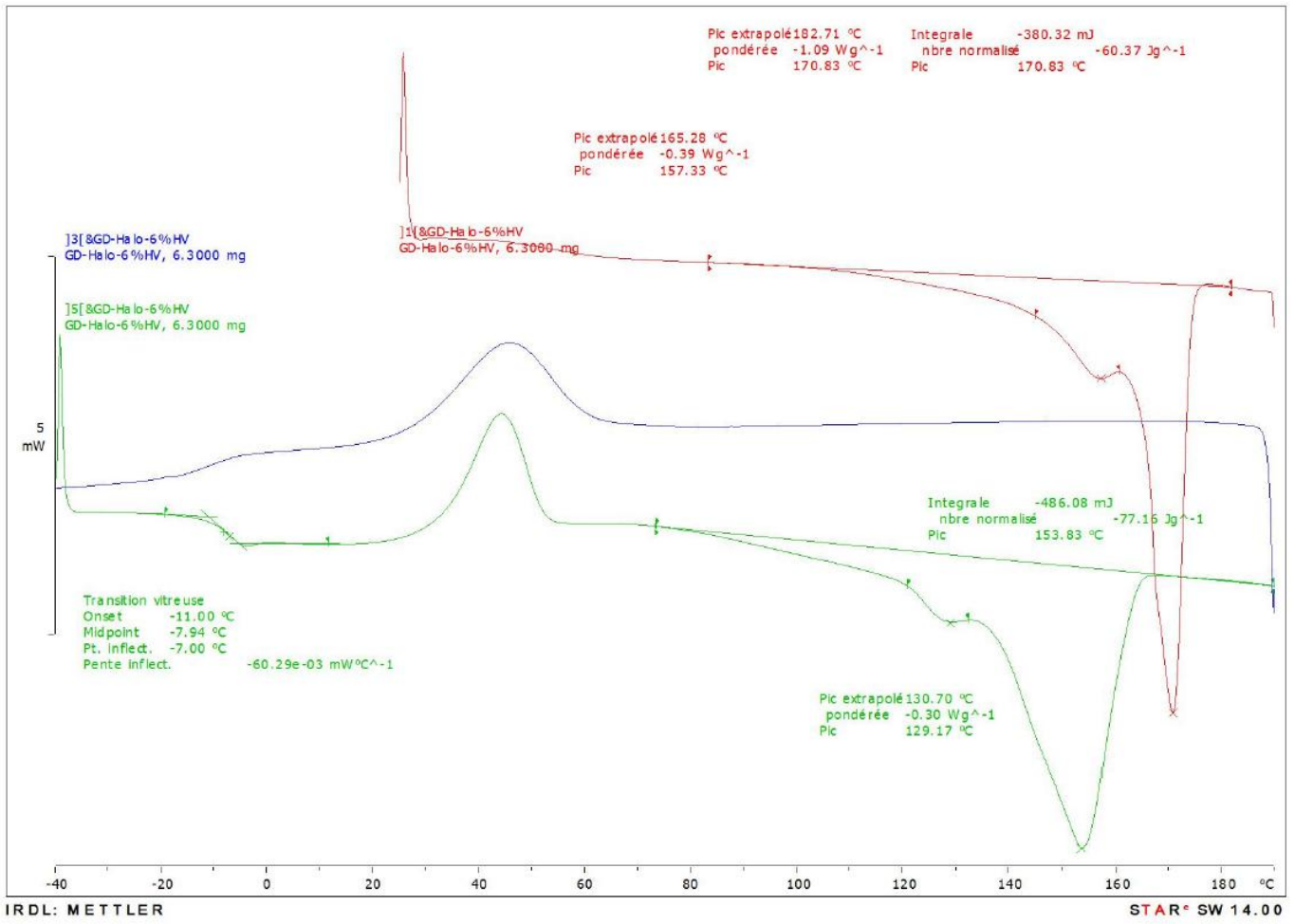


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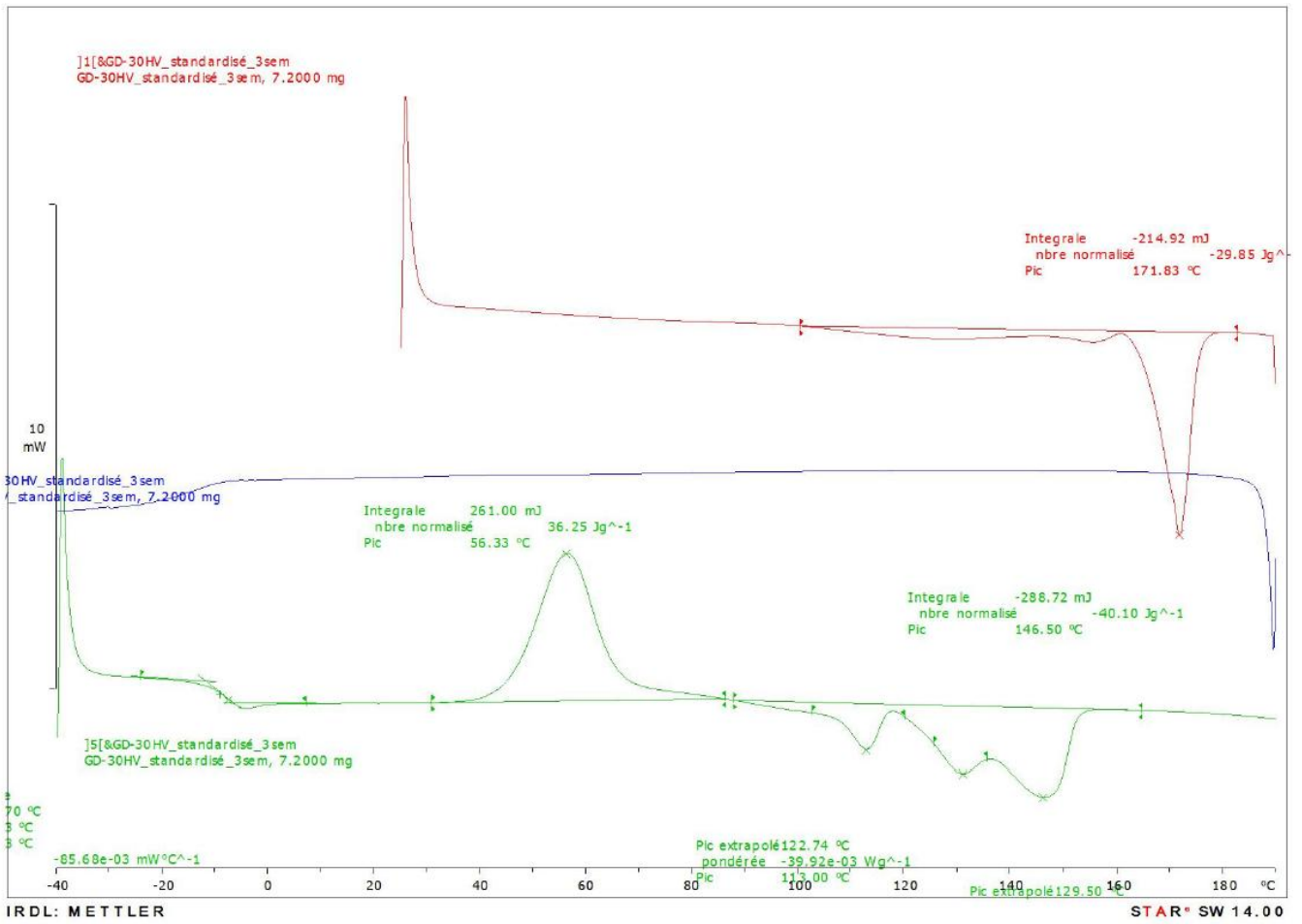
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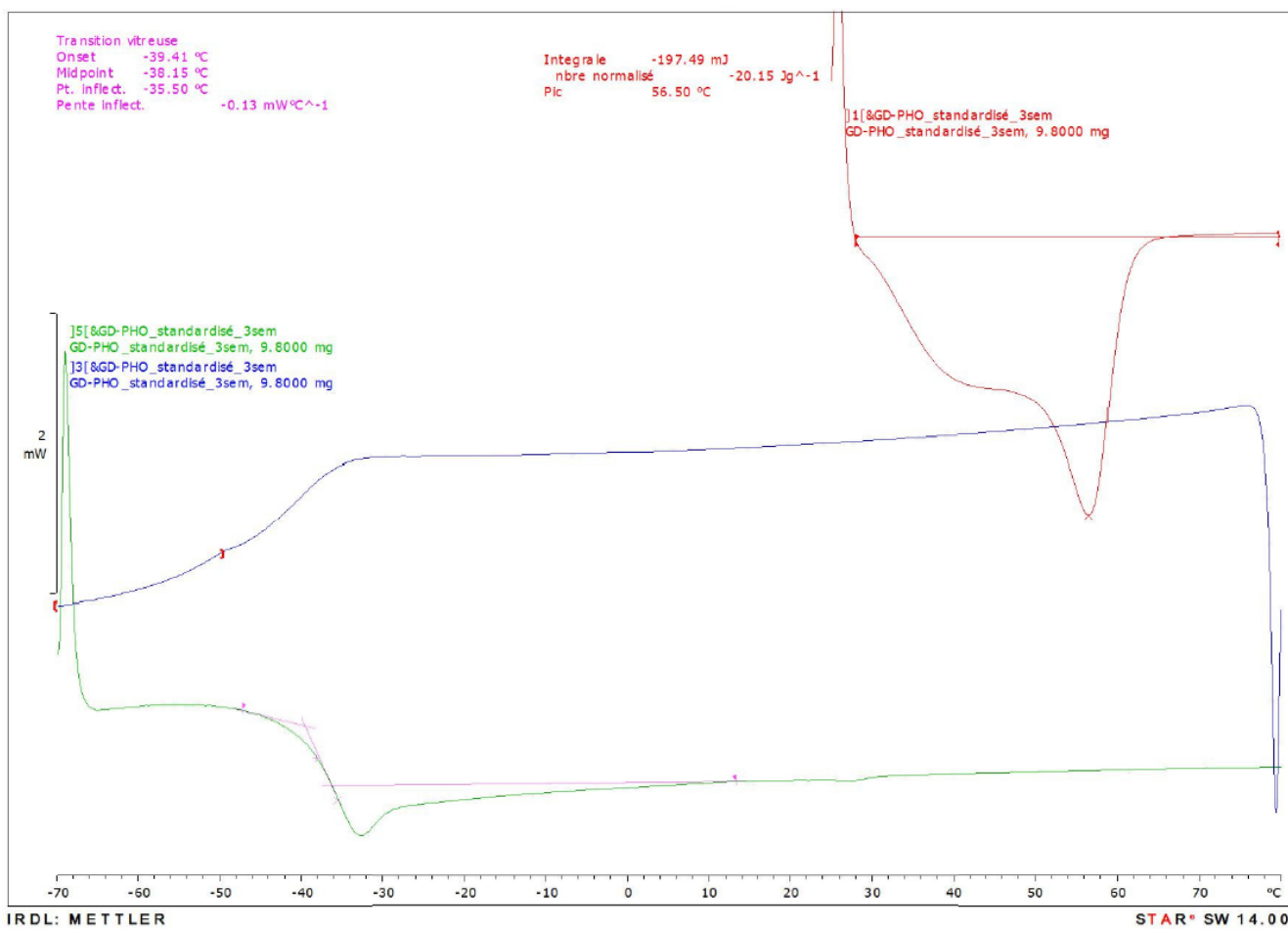
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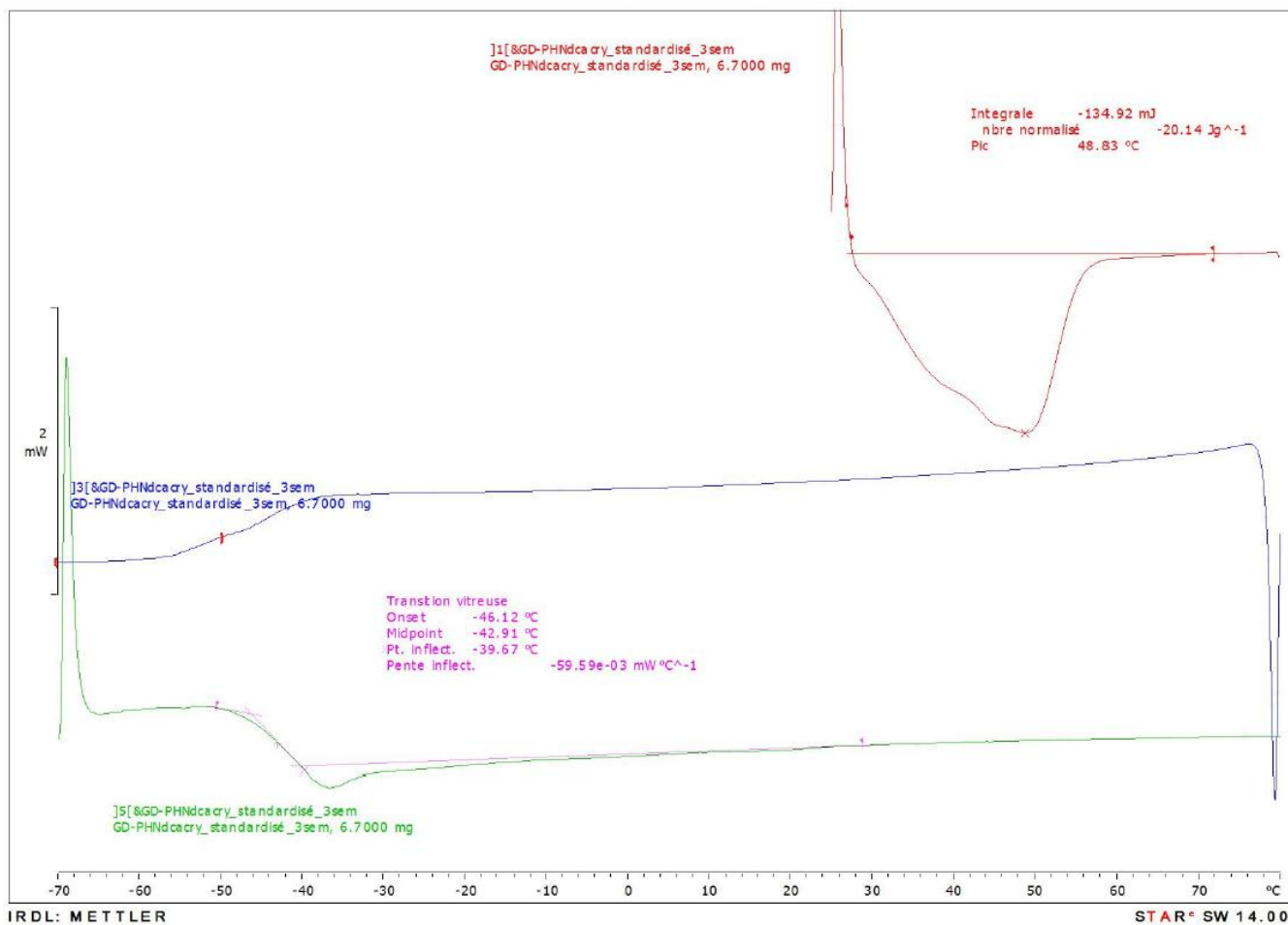


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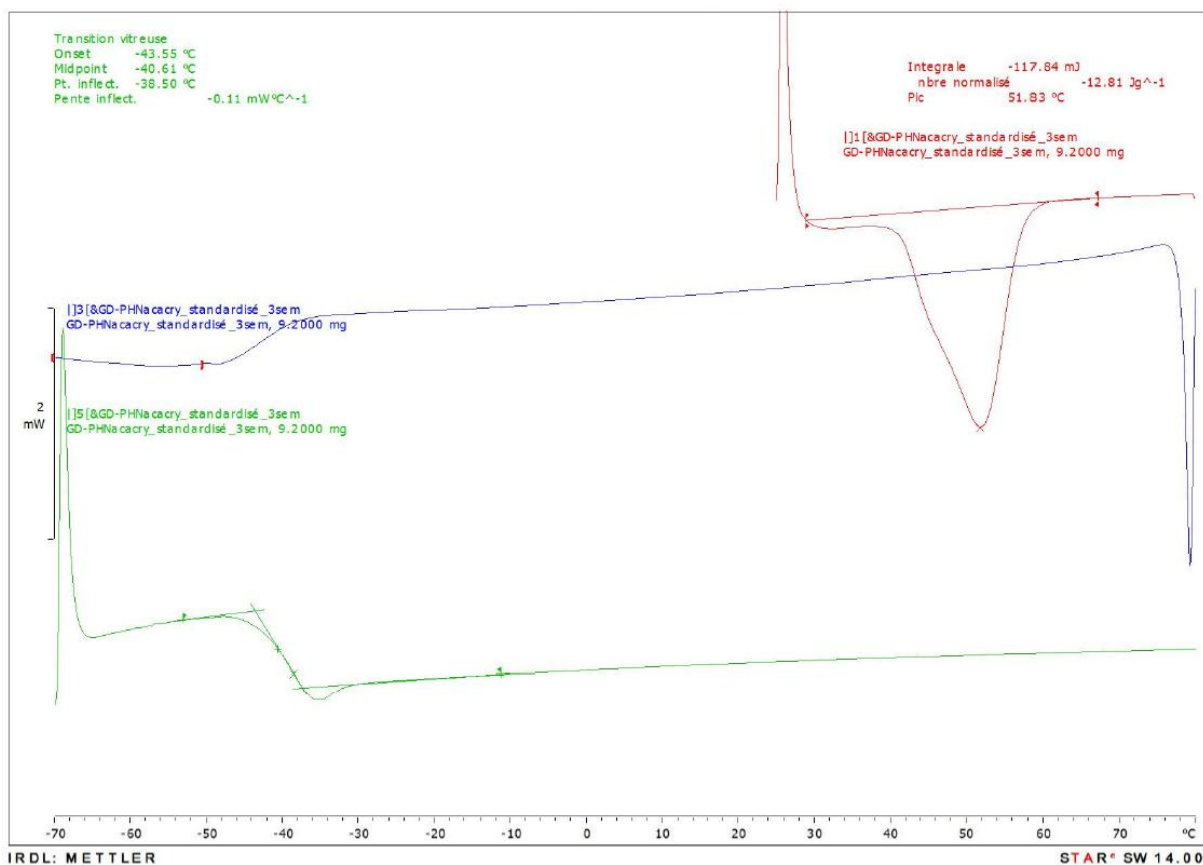
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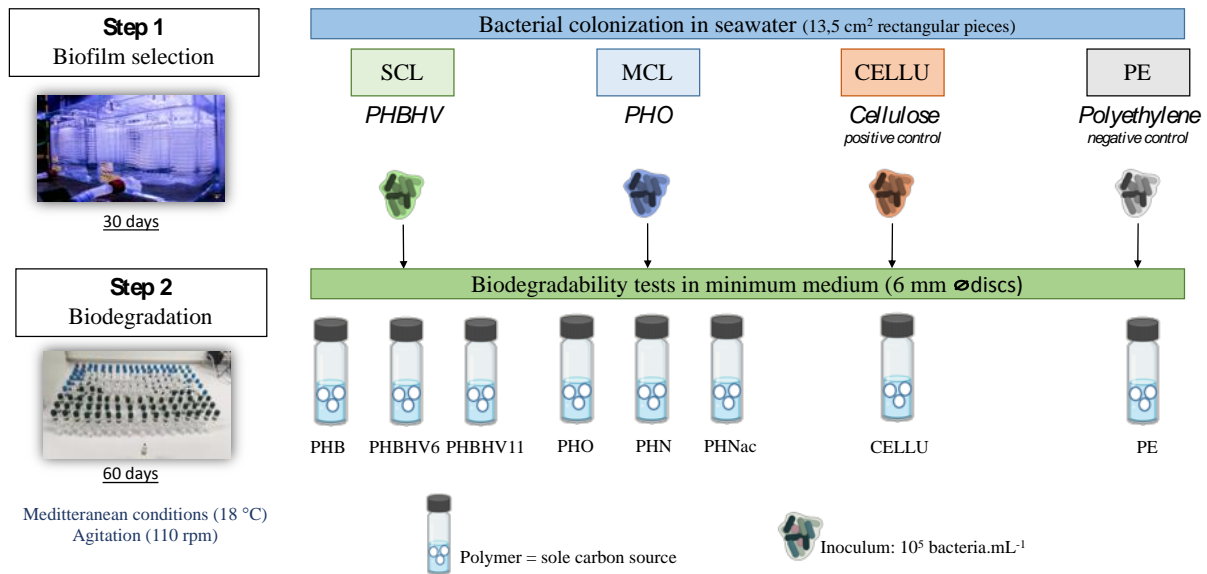
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887 **Appendix B:**

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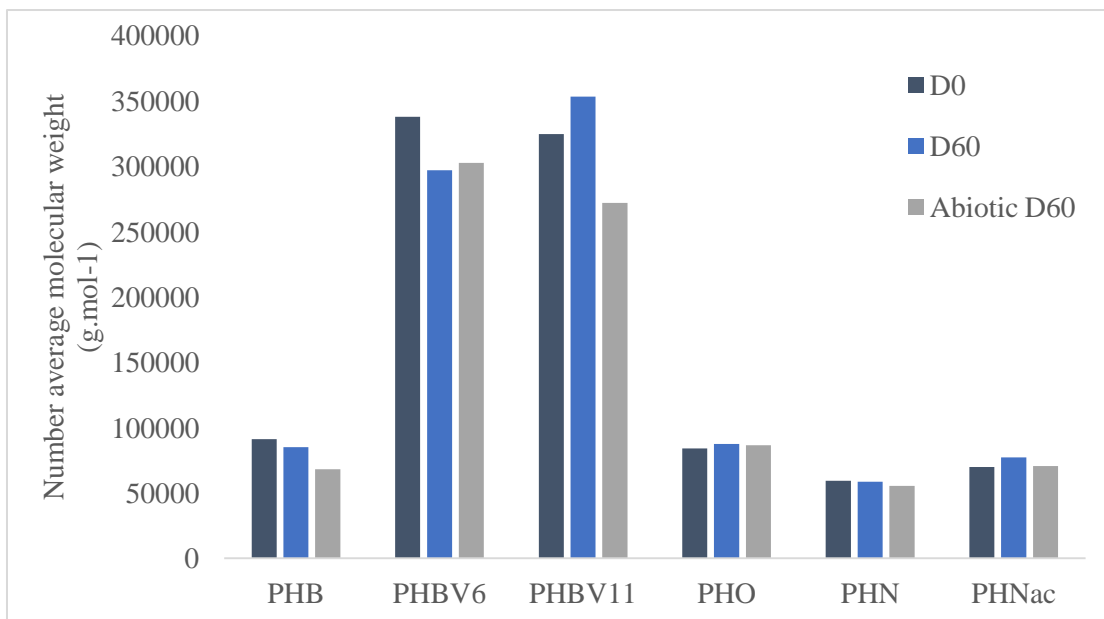
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891 **Appendix C:**

892 Minimal medium composition : : NaCl 24 g.L⁻¹, Na₂SO₄ 4 g.L⁻¹, KCl 0.68 g.L⁻¹, KBr 0.1 g.L⁻¹, H₃BO₃
 893 0.025 g.L⁻¹, NaF 0.002 g.L⁻¹, MgCl₂·6H₂O 10.8 g.L⁻¹, CaCl₂·2H₂O 1.5 g.L⁻¹, SrCl₂·6H₂O 0.024 g.L⁻¹,
 894 NaHCO₃ 0.2 g.L⁻¹, NaHPO₄ 0.04 g.L⁻¹, NH₄Cl 0.5 g.L⁻¹, FeCl₃ 4 g.L⁻¹, EDTA 2 g.L⁻¹, 1 mL of traces
 895 elements for 1 L of medium composed of: CuCl₂·2H₂O 0.015 g.L⁻¹, NiCl₂·H₂O 0.025 g.L⁻¹,
 896 Na₂MoO₄·2H₂O 0.025 g.L⁻¹, ZnCl₂ 0.07 g.L⁻¹, MnCl₂·4H₂O 0.1 g.L⁻¹, CoCl₂·6H₂O 0.12 g.L⁻¹ and 1 mL
 897 of a vitamin solution for 1 L of medium composed of: p-aminobenzoic acid 0.005 g.L⁻¹, pyridoxine-
 898 HCl 0.1 g.L⁻¹, thiamine-HCl 0.05 g.L⁻¹, riboflavin 0.05 g.L⁻¹, nicotinic acide 0.05 g.L⁻¹, D-Ca-
 899 pantothenate 5.10⁻⁹ g.L⁻¹, lipoic acid 0.05 g.L⁻¹, nicotinamide 0.05 g.L⁻¹, B12 vitamin 0.05 g.L⁻¹,
 900 biotine 0.02 g.L⁻¹ and folic acid 0.02 g.L⁻¹.

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902 **Appendix D :** Evolution of the number average molecular weight (g.mol⁻¹) at Day 0, Day 60 and of
 903 abiotic control (PHA but no bacteria) at Day 60.



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