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Biodegradation of polyvinyl chloride plastic films by enriched anaerobic marine consortia

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1 **Biodegradation of polyvinyl chloride plastic films by enriched anaerobic**
2 **marine consortia**

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23

24

25 **Abstract**

26

27 Plastics remarkably contribute to marine litter, which is raising serious concerns. Currently, little
28 is known about the fate of most plastics entering the marine environment and their potential
29 biodegradation rate and extent under anoxic conditions.

30 In this work, biodegradation of polyvinyl chloride (PVC) films by consortia enriched from
31 marine samples (litter and water) was evaluated in anaerobic microcosms. After 7 months, three
32 microcosms showed dense biofilms on plastic surfaces, gravimetric weight losses up to $11.7\pm 0.6\%$,
33 marked decreases in thermal stability and average molecular weight of the polymer, suggesting
34 microbial attack towards polymer chains. After 24 months, further three consortia showed the same
35 abilities. Microbial communities analyzed at month 24 included taxa closely related to those
36 previously reported as halogenated organic compounds degraders. The study is the first report on
37 PVC biodegradation by marine anaerobic microbes and provides insights on potential
38 biodegradation of the plastic film introduced into the sea by native microbes.

39

40 **Keywords:** polyvinyl chloride, plastics colonization, anaerobic marine environment, biodegradation,
41 microbial consortia, microcosms, community composition, dehalogenation.

42

43

44 **1. Introduction**

45 In 2018, global plastic production was 359 million tons, 61.8 of which were produced in Europe
46 (PlasticsEurope, 2019). Over one third of plastics is used to produce disposable products, such as
47 packaging, disposable bags, etc., with a lifespan of 3 years (Gewert *et al.*, 2015; PlasticsEurope,
48 2019; Shah *et al.*, 2008; Wang *et al.*, 2016). From 2006, the amount of plastics wastes that is
49 disposed of in landfill decreased of about 44%, and in 2016 for the first time, recycling overcome
50 landfilling in the EU countries (PlasticsEurope, 2019). Plastic wastes are also widespread in the
51 marine environment where they were found to be the largest debris contaminating marine habitats,
52 with an estimation of more than 250,000 tons (Avio *et al.*, 2017; Oberbeckmann *et al.*, 2016; Wang
53 *et al.*, 2016; Zettler *et al.*, 2013); and between 1.000 and 3.000 tons of floating plastics have been
54 reported in Mediterranean Sea (Avio *et al.*, 2017). This plastic contamination is generated by direct
55 plastic (macro and micro) transfer into aquatic systems or derived from physical/mechanical
56 degradation of macroplastics in landfill that produces microplastics, which are transferred through
57 leachate to rivers and finally to seas and oceans (Avio *et al.*, 2017; Galloway *et al.*, 2017). Further,
58 additives present in plastics formulations are normally not covalently bonded to the polymer chains
59 and could therefore leach out from plastics and enter the marine environment (Avio *et al.*, 2017;
60 Gewert *et al.*, 2015; Harrison *et al.*, 2014). Plastics entering the marine environment could also
61 absorb persistent organic pollutants (POPs) due to the hydrophobic characteristics of these
62 compounds or be a vector agent for the spreading of harmful organisms inhabiting plastic surfaces
63 (Avio *et al.*, 2017; Harrison *et al.*, 2014; Oberbeckmann *et al.*, 2016; Wang *et al.*, 2016; Zettler *et*
64 *al.*, 2013).

65 Among petroleum-based plastics, the PVC is the third one in terms of European plastic demand
66 (5 million tons in 2018) (PlasticsEurope, 2019). PVC plastics have a wide range of applications,
67 such as food packaging, electronics, coatings, medical devices etc., due to low cost, long term
68 stability and mechanical properties depending on quantity and quality of plasticizers added (Bueno-

69 Ferrer *et al.*, 2010; Glas *et al.*, 2014; Reddy *et al.*, 2010). The environmental concern about PVC
70 plastics is due to their high contents of chlorine and additives required for their processing.
71 Additives and stabilizers, e.g. heavy metals or phthalates, provide the necessary physical/chemical
72 and stability properties to the final products but have hazardous characteristics against humans
73 (Glas *et al.*, 2014). Chlorine atoms could produce environmentally harmful chlorinated compounds,
74 HCl or chlorinated dioxins, when disposed of in landfills or incinerated for energy recovery (Glas *et*
75 *al.*, 2014; Reddy *et al.*, 2010).

76 In the last years, there has been an increasing interest in the evaluation of biodegradation of
77 petroleum-based plastics (Ali *et al.*, 2014; Shah *et al.*, 2014; Yang *et al.*, 2015; Tribedi and Dey,
78 2017; Peixoto *et al.*, 2017; Giacomucci *et al.*, 2019; Raddadi and Fava, 2019) with the perspective
79 to develop innovative strategies for mitigating their environmental impact. Considering their
80 hydrophobic nature and the very low bioavailability, the formation of biofilm on the polymer/plastic
81 surface is a critical step necessary for the biodegradation (Das *et al.*, 2012; Reisser *et al.*, 2014). To
82 date, few PVC polymer and plastic biodegradation studies have been reported and were performed
83 mainly under aerobic conditions and in terrestrial systems (Ali *et al.*, 2014; Anwar *et al.*, 2016;
84 Giacomucci *et al.*, 2019; Shah *et al.* 2008; Webb *et al.*, 2000). Marine anaerobic microorganisms
85 have not been studied yet in depth as plastic biodegraders even if they should be the first actors
86 involved in mitigating the marine litter effects associated with the plastics already in the marine
87 ecosystems.

88 The Mediterranean Sea has been identified as a plastic accumulating ecosystem, especially for
89 floating polymeric items (Ruiz-Orejòn *et al.*, 2017; Suaria and Aliani, 2014). Oxygen availability in
90 marine water is decreasing due to climate change with the result of modifying by the expansion of
91 low-oxygen zones, especially in coastal systems (Breitburg *et al.* 2018; Dang and Lovell, 2016).
92 Although most waters in the ocean are oxic and contain aerobic microbes, biofilms on submerged
93 surfaces and marine particles in oxic waters may additionally harbor hypoxic and anoxic
94 microenvironments suitable for microbial anaerobic metabolisms. Hence, anaerobic microbes living

95 in sessile form could be found even in aerobic condition as biofilm structure preserves them from
96 oxygen present in the surrounding environment and thus some bacteria associated to marine fouling
97 need suboxic or anoxic microniches within particles to support microaerophilic or anaerobic
98 metabolism (Celikkol-Aydin *et al.*, 2016; Dang and Lovell, 2016).

99 The aim of the present study was to evaluate the biodegradation of a virgin PVC plastic film
100 under marine anaerobic conditions. For this purpose, 16 anaerobic consortia were enriched from
101 marine samples (water with litter fraction) in the presence of PVC films in laboratory microcosms
102 and their biodegradation abilities were assessed after 7 and 24 months incubation. At the end of the
103 experiment, the PVC film-associated microbial communities were characterized.

104 **2. Materials and Methods**

105 **2.1. Sampling and pre-enrichment of marine anaerobic consortia**

106 Marine samples were collected from four sampling stations (S1 to S4) in Elefsis Bay (Greece) at
107 a depth of 0 to 1.2 m of the water column. The samples, named S1 to S4 and consisting of marine
108 water and a litter/debris fraction, were used as source of microbial communities. To increase the
109 anaerobic microbial biomass to be used as inoculum in biodegradation assays, a pre enrichment step
110 of the microbes adhered to the collected marine litter was performed. Specifically, four anaerobic
111 microcosms from each sampling station were set up, using four different growth media, in 100 ml
112 serum bottles with working volume of 40 ml. For this purpose 4 ml of litter fraction, 16 ml of
113 marine water and further 20 ml of the anaerobic specific medium containing 24 g/L of sodium
114 chloride were mixed. The four culture media (M1, M2, M3 and M4; composition reported in
115 Supplementary material S1) were used in order to try to favour the growth of different anaerobic
116 consortia, i.e. acidogenic/methanogenic (M1 and M2), nitrate reducing (M3) and sulfate reducing
117 (M4) microorganisms. The sixteen different microcosms were flushed with pure sterile nitrogen (in
118 the case of M1, M3 and M4 media) or a mix of nitrogen and carbon dioxide (N₂:CO₂=80:20, for the
119 M2 medium) and incubated at 20°C. Microbial growth was evaluated after 3 months incubation

120 based on gas composition analysis of the headspace of each microcosm (H₂, CO₂, CH₄) by gas
121 chromatography (Agilent 3000 MicroGC, Agilent technologies, Santa Clara, CA, USA) equipped
122 with thermal conductivity detector (TCD). The analytical conditions were as follows: injector
123 temperature 90 °C; column temperature 60 °C; sampling time 20 s; injection time 50 ms; column
124 pressure 25 psi; run time 44 s; carrier gas N₂. When microbial growth was detected, fresh medium
125 was added to a final volume of 70 ml and microcosms were incubated under the same conditions
126 until a significant growth was detected, before using as inocula in the biodegradation experiment.

127 A set of four negative controls with the corresponding non inoculated media were also prepared.

128

129 **2.2. PVC film**

130 PVC film containing a total of 30%_{w/w} of additives was provided by an Italian plastic producing
131 company. In order to better monitor the gravimetric weight loss, thick PVC plastic films (300 µm),
132 obtained after pressing several PVC thin layers (20 µm each) at 17.44 bar and 160°C for 4-5 min,
133 were used. Thick films were then cut into small pieces (1.5 cm × 1.5 cm and 1.5 cm × 3.2 cm),
134 degreased and sterilized by 30 min immersion in 70%_{v/v} ethanol solution and washed with sterile
135 distilled water before use. The films used for the determination of gravimetric weight loss were
136 dried under vacuum to constant weight before being used as substrate for microbial growth.

137

138 **2.3. PVC biodegradation assays**

139 The sixteen microbial communities from the pre-enrichment step were used as inocula for PVC
140 biodegradation experiments (Figure S1). Specifically, sixteen microcosms were setup in 100 ml
141 serum bottles with 50 ml of the corresponding medium containing 10 g/l of PVC films as major
142 carbon and energy source and inoculated with 20%_{v/v} of pre-enriched cultures. Inoculated
143 microcosms and abiotic controls were incubated under static conditions at 20°C. Samples of plastic

144 films and culture broth were withdrawn after 7 and 24 months incubation and subjected to the
145 following analyses in order to detect putative microbial growth and PVC films biodegradation.

146 *2.3.1. Evaluation of the microbial growth and colonization of the PVC film surface*

147 Viability and growth of anaerobic consortia were evaluated by measuring headspace gas
148 production and composition (H₂, CO₂, CH₄) using MicroGC-TCD.

149 Biofilm formation was assessed by quantifying the proteins available onto the PVC film
150 surfaces. For this purpose, sampled PVC films were incubated overnight (4°C, 150 rpm) in a 6 M
151 urea solution. Protein quantification according to Lowry method (Lowry *et al.*, 1951) was then
152 performed on urea solutions, and urea 6 M was used as blank.

153 *2.3.2. Evaluation of PVC films biodegradation*

154 *Gravimetric weight loss.* Measurement of the dry weight of PVC film was determined after biofilm
155 removal by incubation with a 6 M urea solution overnight at 4°C and further washing with distilled
156 water. The washed PVC films were dried under vacuum at room temperature to constant weight (at
157 least 24 h) before weighing using a balance with a 6 digit accuracy. The weight loss percentage was
158 calculated as follows:

$$159 \text{ Weight loss (\%)} = (\text{Initial weight} - \text{Final weight}) / \text{Initial weight} * 100.$$

160 *Thermogravimetric analysis (TGA).* Dried plastic samples of 5 to 10 mg were subjected to TGA
161 using a Perkin Elmer TGA7 thermal analyzer under nitrogen atmosphere (gas flow: 40 ml/min).

162 The thermograms were recorded from 40°C to 800°C at a heating rate of 10°C/min. The onset
163 degradation temperature (T_{onset}) and the maximum degradation temperature (T_{max}) were noted. Non
164 incubated PVC film and PVC resin were used as reference.

165 *Gel permeation chromatography (GPC).* The molecular weight of the PVC films were determined
166 using GPC. Measurements were carried out on a HPLC Lab Flow 2000 equipped with Phenomenex
167 Phenogel Mixed 5µ MXM/MXL columns and a Linear Instrument UVIS-200 detector operating at
168 254 nm. Tetrahydrofuran was used as mobile phase (1 ml/min) after calibration with polystyrene

169 standards of known molecular mass. A sample concentration of 3 mg/ml was applied. Non
170 incubated PVC film and PVC films incubated under abiotic conditions were used as references.

171 2.3.3. *Statistical analyses*

172 Results of gravimetric weight losses and GPC analyses were statistically evaluated using one-way
173 ANOVA. Post-hoc Tukey test was applied to determine whether gravimetric or molecular weight
174 changes of PVC films showed significantly different extents. Statistically significant results were
175 depicted by *p-values* < 0.05. All the analyses were performed using GraphPad Prism version 8.0.0
176 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

177

178 2.4. Composition of planktonic and biofilm communities

179 After 24 months incubation, total DNA was extracted using a Powersoil[®] DNA isolation kit (MOBIO,
180 Carlsbad, CA, USA) from planktonic (from 2 ml of culture broth) and biofilm (PVC adhered
181 microbial cells) microbial communities. Bacterial partial 16S rRNA gene was amplified using 357F-
182 GC and 907R primers and PCR conditions reported by Polo *et al.* (2010) except for MgCl₂ and dNTP
183 mix concentration of 1.8-2 mM and 0.2 μM, respectively and annealing temperature of 55°C.
184 Archaeal PCRs were performed using Arch344F-GC and Arch918R (Giovannoni *et al.*, 1988; Stahl
185 and Amann, 1991) primer pair. The archaeal thermal protocol was a modification of the bacterial
186 protocol by elongating each step of 15 sec. DGGE was according to Polo *et al.* (2010), using
187 denaturant gradients of 40–60% and 30–70% for bacterial and archaeal communities, respectively.
188 Principal bands were excised, eluted in 50 μl milliQ water (30°C for 3 h), amplified and identified by
189 sequencing (Macrogen Inc., Republic of Korea). The sequences were checked for chimeras using
190 DECIPHER software (Wright *et al.*, 2012), analyzed using the BLASTN software and submitted to
191 the Genbank database (accession numbers MH520751 to MH520766).

192

193 3. Results and discussion

195 3.1. PVC films biodegradability after 7 months

196 3.1.1. Microbial growth monitoring

197 Before performing the biodegradation assays, a pre-enrichment step (growth in the absence of
198 PVC film) was performed (please refer to the scheme of the experimental design in supplementary
199 Figure S1) and microbial growth was confirmed by headspace gas analysis. The pre-enriched
200 consortia were used as inocula in the biodegradation assays. Microbial growth in the presence of
201 PVC films as major carbon and energy source was monitored by measuring the volume and
202 composition of the gas produced in the headspace of each consortium. After 7 months incubation,
203 significant gas volumes were measured in 10 microcosms (Figure 1A) reaching a maximum of
204 19.0 ± 1.0 ml, while no gas production was detected in the four abiotic controls. Headspace gas
205 analysis showed that CO₂ was produced in all the microcosms while hydrogen was recorded only in
206 the case of S2-M1 consortium (5.8 ± 0.0 ml) (Figure 1A). Methane (max volume = 5.7 ± 0.1 ml) was
207 produced by the four consortia enriched in M2 medium, while very low/no methane production was
208 recorded in microcosms enriched in M1, M3 and M4 media. These results showed that growth of
209 the native marine microbial communities occurred in the presence of PVC plastic film as major C
210 and energy source. Plasticizers probably sustained the observed growth, in line with previous
211 studies carried out with PVC plastic under sulfate reducing conditions with bacteria enriched from
212 landfill (Tsuchida *et al.*, 2011).

213 Based on these results, the 10 microcosms displaying clear microbial growth on PVC films (S2-
214 M1, S2-M2, S3-M2, S4-M2, S1-M3, S3-M3, S4-M3, S2-M4, S3-M4 and S4-M4) were selected for
215 further analysis.

216 3.1.2. Evaluation of microbial colonization of PVC film surface.

217 Among PVC films incubated in the 10 microcosms, four showed significantly higher (>10 times)
218 amount of adhered proteins compared to their corresponding abiotic controls (Figure 1B),

219 suggesting an extensive microbial colonization of plastic surface. These include S2-M2, S3-M2, S4-
220 M3 and S2-M4, with a protein content (mean \pm SD) of 57.3 \pm 2.9, 45.6 \pm 2.3, 88.9 \pm 4.4 and 76.3 \pm 3.8 μ g
221 of BSA equivalent/mg of PVC film, respectively. Microbial colonization of PVC films was reported
222 under aerobic conditions. Specifically, untreated plasticized PVC films exposed to the
223 atmosphere (Blackley, Manchester, United Kingdom) for 95 weeks were found to be colonized by
224 fungi while no bacterial colonization was recorded (Webb *et al.*, 2000). Dang *et al.* (2008)
225 evaluated biofilm formation on different polymer surfaces including PVC plates, submerged in
226 seawater (1 m below the water surface) near the Qingdao coast for 72 h. Biofilm formation was
227 shown to occur on the different surfaces based on detection of 126 operational taxonomic units with
228 prokaryotes being predominant.

229 To the best of our knowledge, to date there is only one report on microbial colonization of PVC
230 plastic surface under anaerobic conditions. The Archaeal strain *Methanosarcina barkei* was found
231 to be able to adhere on PVC films after 2 h of exposure and to putatively produce exopolymeric
232 substances (EPS), as revealed by epifluorescence and scanning electron microscope observations
233 (Nguyen *et al.*, 2016).

234 Biofilm formation is considered as the first step of biodegradation activity against water
235 insoluble polymeric materials (Das *et al.*, 2012; Reisser *et al.*, 2014; Wang *et al.*, 2016; Zettler *et*
236 *al.*, 2013). Hence, only microbial consortia that showed significantly high adhered protein contents
237 on PVC films, i.e. S2-M2, S3-M2, S4-M3 and S2-M4 (Figure 1B), were selected for further
238 analyses. Indeed, for the PVC films incubated in these microcosms, a 10-fold protein content was
239 recorded compared to the corresponding abiotic controls. This threshold was fixed in order to
240 overcome the limitations of the Lowry assay related to the interference of molecules other than
241 proteins (like compounds that derive from the additives components of the plastic film), in the
242 colorimetric measurements (Everette *et al.*, 2010).

243 3.1.3. Evaluation of PVC biodegradation

244 Biodegradation activity was evaluated on film samples withdrawn from the four (S2-M2, S3-M2,
245 S4-M3 and S2-M4) selected microcosms. Gravimetric measurements revealed a statistically higher
246 decrease in their weights ($p < 0.05$; Figure 2 A-D) compared to those recovered from the
247 corresponding abiotic controls. Specifically, weight reductions (mean \pm SD%) of $11.67\pm 0.58\%$,
248 $10.71\pm 0.54\%$, $6.26\pm 0.31\%$ and $5.77\pm 0.29\%$ were recorded for the films incubated in S4-M3, S2-
249 M4, S2-M2 and S3-M2 microcosms, respectively. Among the biological effect of the four selected
250 consortia, the highest gravimetric weight decrease was recorded by films incubated with S4-M3 and
251 S2-M4 consortia, which showed the same statistical film weight reduction followed by the action of
252 S2-M2 and S3-M2 consortia ($p < 0.05$; Figure 2 D). The corresponding abiotic controls incubated in
253 the growth media M3, M4 and M2 showed statistically equal weight losses of $3.71\pm 0.28\%$,
254 $3.91\pm 0.2\%$ and $3.51\pm 0.18\%$ respectively ($p < 0.05$; Figure 2 A-D) due to additives leaching from
255 the PVC films (Kastner *et al.*, 2012; Suhrhoff and Scholz-Bottcher, 2016; Wang *et al.*, 2016).
256 These evidences are the first proof of plastic biodegradation; indeed, the reduction of gravimetric
257 weight, which is a direct evidence of the loss of material, is widely used in biodegradation tests
258 (Shah *et al.* 2008; Wang *et al.*, 2016). Thus some components of the PVC films were degraded by
259 the enriched microbial communities. Since the films used in this study had about 30%_{w/w} of
260 additives, further analyses were performed in order to elucidate if the biodegradation regarded the
261 sole additives or also the PVC polymer.

262 TGA showed a lower thermal stability of the films recovered from the four active microcosms
263 (Figure 2A-C) compared to those incubated under abiotic conditions. Specifically, a T_{max} ranging
264 from 287°C to 277°C was recorded for the former compared to a T_{max} of 292°C for those of abiotic
265 controls or 294°C for the non incubated PVC film. Considering that the presence of additives
266 (citrates, polyadipates, epoxidized soybean oil, Zn etc; data supplied by the production company) is
267 lowering the thermal stability of PVC resins once in PVC plastic film, the (bio)degradation of the
268 sole additives would lead to an increase of the film thermal stability i.e. a shift of the TGA curve

269 toward that of the resin. On the opposite, here a reduction of such parameter was observed and
270 would suggest that a microbial attack towards PVC polymer chains occurred.

271 Also GPC analyses exhibited a decrease of the average molecular weight (M_n %; mean \pm SD) from
272 100% to 91.1 \pm 3.2%, 94.3 \pm 3.3%, 95.5 \pm 3.3%, and 98.1 \pm 3.4% for PVC films incubated with S4-M3,
273 S3-M2, S2-M2, and S2-M4 consortia, respectively (Figure 2E). According to statistical analysis,
274 PVC film incubated with S4-M3 exhibited a statistically lower M_n (%) compared to the film
275 incubated with its abiotic control ($p < 0.05$, Figure 2E), suggesting that this consortium was able to
276 statistically reduce PVC molecular weight. On the other hand, no statistically significant differences
277 were recorded between molecular weights of films incubated with all consortia and pristine PVC
278 film ($p > 0.05$; Figure 2E). However, the fact that the differences between biotically treated and
279 pristine PVC films are not statistically significant does not imply that no biological action has
280 occurred and the interpretation of the results should focus on the scientific importance rather than
281 on the statistical significance. Indeed, considering the nonbiodegradable nature of the polymer even
282 small changes that are not statistically significant are important from a biodegradation view point.
283 Hence, the GPC results are in line with those of TGA and suggest that some polymer chains were
284 biodegraded with the production of low M_w PVC chains in the case of S4-M3, S3-M2 and S2-M2
285 microcosms (Figure 2E). On the other hand, GPC analyses revealed an increase of M_n of abiotic
286 controls up to 105.5 \pm 3.7% (mean \pm SD; Figure 2E) although no appreciable changes in their thermal
287 stability were recorded. Such results could be explained by a loss of water-soluble low molecular
288 weight additives other than thermal stabilizers, which has led to M_n increase without change in the
289 thermal stability.

290

291 **3.2. PVC films biodegradation after 24 months**

292 Figure 3 shows the gravimetric weight reductions of the PVC films incubated in the 16
293 biologically active microcosms or in the corresponding abiotic controls after 24 months incubation.
294 According to statistical analysis, eleven consortia (three in M2, 4 in M3 and 4 in M4 medium) were

295 shown to significantly reduce film weight compared to their abiotic controls ($p < 0.05$; Figure 3).
296 Four and three consortia enriched in M4 and M3 media reduced the gravimetric weight (mean \pm SD)
297 of the PVC films by up to $12.97\pm 0.65\%$ ($p < 0.05$; showed by d letter in Figure 3). A statistically
298 lower weight loss of up to $9.23\pm 0.46\%$ ($p < 0.05$; showed by c and e letters in Figure 3) was
299 recorded for three microcosms set up in M2 and for S2-M3 consortium, while a maximum weight
300 loss of $4.21\pm 0.21\%$, statistically comparable to that recorded under abiotic conditions
301 ($4.32\pm 0.22\%$), was observed for the four consortia enriched in M1 and in S4-M2 consortium ($p <$
302 0.05 ; Figure 3).

303 Based on these results, only 11 consortia (S1-M2, S2-M2, S3-M2, S1-M3, S2-M3, S3-M3, S4-
304 M3, S1-M4, S2-M4, S3-M4 and S4-M4), exhibiting statistically significant gravimetric weight
305 losses ($p < 0.05$), were selected for further analyses.

306 Compared to abiotic control and pristine (non-incubated) PVC films, the films withdrawn from
307 S2 and S3 consortia enriched in M2, the S4 consortium enriched in M3 as well as S1, S2 and S4
308 consortia enriched in M4 displayed a decrease of TGA-assessed thermal stability (Table 1),
309 confirming the evidences observed after 7 months incubation.

310 On the other hand, the GPC analyses showed an increase of the M_n (%) of the films incubated
311 with 8/11 consortia (S2-M2, S3-M2, S2-M3, S4-M3, S1-M4, S2-M4, S3-M4 and S4-M4) including
312 those that exhibited M_n (%) lower than 100% at month 7, even if no statistically significant
313 differences were found among M_n (%) after the analysis of variance (ANOVA: $p = 0.19$; $F = 1.44$;
314 $df = 14$) (Figure 2E, Figure 4). Once again, the fact that no statistically significant differences in M_n
315 (%) were observed does not imply no modifications of the M_n (%) has occurred. Indeed, it is
316 important to notice that M_n values for polymeric substances are very high numbers and therefore
317 differences in such number should be very high to be considered statistically different.
318 Consequently, in the case of data obtained from a biodegradation of materials known as “non
319 biodegradable”, small differences in M_n (%) are often observed and should be acknowledged as of
320 scientific importance rather than of statistical significance. Hence, these results suggest that the

321 three consortia found to be the most active at the 7th month together with consortia S1, S2 and S4
322 enriched in M4 that displayed activity after longer incubation period, are apparently able to degrade
323 PVC plasticizers and polymer chains. Indeed, considering that biodegradation of low molecular
324 weight compounds affects prevalently the M_n (%) rather than the gravimetric weight, the M_n
325 increase observed after 24 months incubation could be explained by the full biodegradation of the
326 short polymer chains putatively produced within the first months of incubation and/or the release of
327 the broken chains and of the lower molecular weight additives into the growth medium.

328

329 **3.3. Characterization of microbial communities**

330 Characterization of the anaerobic communities enriched in the presence of PVC films was
331 performed on the 11 different consortia exhibiting a mass reduction significantly higher than the
332 corresponding abiotic controls after 24 months incubation.

333 From the DGGE profiles (Figure 5), 13 (band #1 to #13) and 3 (band #A to #C) bands were cut
334 from the bacterial and archaeal communities, respectively.

335 The bacterial biofilm communities enriched in M2, M3 and M4 media were characterized by the
336 presence of two bands that were detected in most consortia and which include band #8 and band
337 #10 detected in 9 and 8 out of the 11 consortia, respectively (Figure 5, Table 2). The band #8 has
338 highest sequence similarity with different Clostridia including uncultured *Clostridium* clone
339 reported from an Alaskan mesothermic petroleum reservoir (Pham *et al.*, 2009), Clostridiales
340 bacteria able to degrade the explosive UXO5-23 (Zhao *et al.*, 2007) or *Clostridium* sp. AN-D
341 reported from high pressure prokaryotic enrichments from deep marine gas hydrate sediments
342 (Parkes *et al.*, 2009). The band #10 has 98% similarity with *Acetobacterium* sp. Other two bands
343 (band #9 and band #7) which have, respectively, 100% and 99% of similarity with
344 *Dethiosulfovibrio* sp. and *Sporobacter* sp. were detected in 3/11 consortia (Figure 5, Table 2).
345 Different other bands were detected in some of the communities enriched in specific media and
346 having homologies with: i) *Erysipelothrix* sp. (band #1) mainly in M4 communities; ii) *Fusibacter*

347 (band #13) and *Psychromonas* (band #4) in some M3 communities and iii) *Desulfovibrio* (band
348 #12), *Cupriavidus* (band #5), *Cohaesibacter* sp. (band #11) and *Pleomorphochaeta* (band #6)
349 detected mainly in M2 consortia (Figure 5, Table 2).

350 For all the consortia, almost no differences in the composition of the bacterial biofilm and its
351 corresponding planktonic community were observed. Similarly, composition of the planktonic and
352 biofilm archaeal communities for each consortium were very similar. They were composed of bands
353 having high similarities with i) *Methanosaeta* sp. (band A) present in all M4 and M3 communities,
354 ii) *Methanococoides* sp. (band B) detected in the three M2 communities and iii) *Methanogenium*
355 sp. (band C) found only in some M2 communities (Figure 5, Table 2).

356 Although the chemical analyses indicate the biodegradation of PVC polymer by the consortia
357 S2-M2, S3-M2, S4-M3, S1-M4, S2-M4 and S4-M4, none common microbial phylotype was
358 observed in all such consortia and this seems to suggest that more than a microbial specie were
359 involved in the biodegradation. The occurrence of high NaCl contents in the microcosm media did
360 not allow to determine changes in chlorine ion concentration in the culture supernatants and
361 therefore to assess if the degradation was accompanied by the dehalogenation of PVC polymer.
362 However, the following observations support PVC dechlorination. Among the microbial
363 components of two communities (S1-M2; S1-M3), bands having a high similarity with the
364 facultative organohalide respiring bacterium *Desulfovibrio dechloracetivorans* (band #12, S1-M2)
365 (Sun *et al.*, 2000) and with *Fusibacter* sp. (band #13, S1-M3) were detected. *Fusibacter* sp. was
366 reported as dominant component of tidal flat communities where *Dehalococoides*-like bacteria
367 were not detected although dechlorination of PCE-to-cis-DCE has occurred (Lee *et al.*, 2011).
368 Further, bands having highest sequence similarity with Clostridia were detected in almost all
369 consortia. The class of Clostridia includes bacterial species (bacteria from the genus *Clostridium*)
370 reported to play an important role in dechlorinating consortia, producing H₂ that is used as electron
371 donor by the dechlorinating bacteria (Bowman *et al.*, 2010).

372 Bacteria of the genus *Acetobacterium* were detected in 8/11 consortia. Among these
373 homoacetogens, some species have been reported to be able to mineralize tetrachloromethane (Egli
374 *et al.*, 1988). *Acetobacterium* spp. was also detected among the components of consortia able to
375 dechlorinate 1,1,2,2-tetrachloroethane (Manchester *et al.*, 2012). Patil *et al.* (2014) reported
376 successful dechlorination of tetrachloroethene by a microbial community that did not include
377 classical dechlorinators due to the synergistic work of the community i.e. *Enterobacter* and
378 *Desulfovibrio* spp. as putative dechlorinators and *Bacteroidetes* and *Firmicutes* as acetate
379 fermenters (Patil *et al.*, 2014).

380 Other bands having high similarity with Bacteroidales bacterium CF (band#3, 93% of identity),
381 were also detected. This bacterium was found to be the most abundant non dechlorinating bacterium
382 in a community that reductively dechlorinates multichlorine molecules; it is able to ferment lactate,
383 ethanol and glucose in the presence of concentrations of chloroform or trichloroethane that usually
384 inhibit reductive dechlorination and methanogenesis (Tang *et al.*, 2013).

385 The archaeal communities present in 8/11 consortia enriched in M3 and M4 media were
386 composed mainly by members of the Phylum Euryarchaeota, with a unique member of the
387 Methanosarcinales (Genus *Methanosaeta*, species *pelagica*, Figure 5, Table 2) known for its
388 acetoclastic methanogenic activities. Oh *et al.* (2008) showed that acetoclastic methanogenic
389 Archaea may play an indirect role in dechlorination of halogenated organic compounds such as
390 PCBs by synthesizing the corrinoid cofactor cobalamin required for vitamin B12 synthesis, which is
391 necessary for assembling functional reductive dehalogenase systems (Patil *et al.*, 2014; Rupakula *et*
392 *al.*, 2014; Yan *et al.*, 2013). Cobalamine and other precursors are typically produced by
393 methanogenic Archaea as cofactors for methyltransferases involved in methane production (Yan *et*
394 *al.*, 2013).

395 Specialized dehalorespiring bacteria among the components of the anaerobic consortia studied
396 were, however, not detected. This could be due to PCR bias i.e. the necessity to use more specific

397 amplification primers in order to reveal the presence of such bacteria (Aulenta *et al.*, 2004;
398 Dowideit *et al.*, 2010; Duhamel *et al.*, 2006; Manchester *et al.*, 2012).
399 Besides of the bacterial and archaeal phyla potentially playing an important role in dechlorinating
400 PVC, the other bacterial and archaeal clades were detected and could be involved in additives
401 biodegradation. These include *Dethiosulfovibrio* sp.; *Erysipelothrix* sp.; *Sporobacter* sp.;
402 *Psychromonas* sp.; *Cupriavidus* sp. and *Cohaesibacter* sp. (Bonin *et al.*, 2002; Canion *et al.*, 2013;
403 Diels *et al.*, 2009; GrechMora *et al.*, 1996; Hwang *et al.*, 2008; Pantazidou *et al.*, 2007; Stackebrandt
404 *et al.*, 2006; Surkov *et al.*, 2001) as well as *Methanococcoides* sp. (99% of identity, Figure 5, Table
405 2) detected in three M2 consortia or *Methanogenium* sp. detected only in S1-M2 consortium (Figure
406 5, Table 2) (Miller *et al.*, 2016).

407

408 **4. Conclusions**

409 A significant biodegradation of plasticized unpretreated PVC films by marine anaerobic
410 microbial consortia was demonstrated in this study. After 7 months incubation, three of 16 enriched
411 consortia were able to degrade the films apparently acting against both the additives and the
412 polymer chains. For these consortia, GPC showed a decrease in M_n suggesting that some polymer
413 chain scission took place leading to the formation of smaller fragments, while TGA and gravimetric
414 weight loss (%) revealed a decrease in thermal stability and a mass loss. Prolongation of the
415 incubation to 24 months allowed the selection of other three consortia able to display the same
416 potential.

417 To our knowledge, this is the first study reporting on biodegradation of plasticized PVC films by
418 marine microbial communities enriched under anoxic conditions and providing information about
419 the composition of such communities potentially responsible for it. The PVC film-associated
420 communities of the biodegrading microcosms encompasses microbial phyla closely related to those

421 previously reported from laboratory/field enrichments for their ability to degrade halogenated
422 organic compounds and hydrocarbons.

423 The enriched consortia originate from marine samples and the activity recorded was obtained
424 under lab conditions mimicking the sulfate reducing, nitrate reducing and methanogenic conditions
425 occurring in marine environment. Thus the present study provides relevant insights on the potential
426 fate of PVC plastic films occurring in anoxic marine impacted ecosystems.

427

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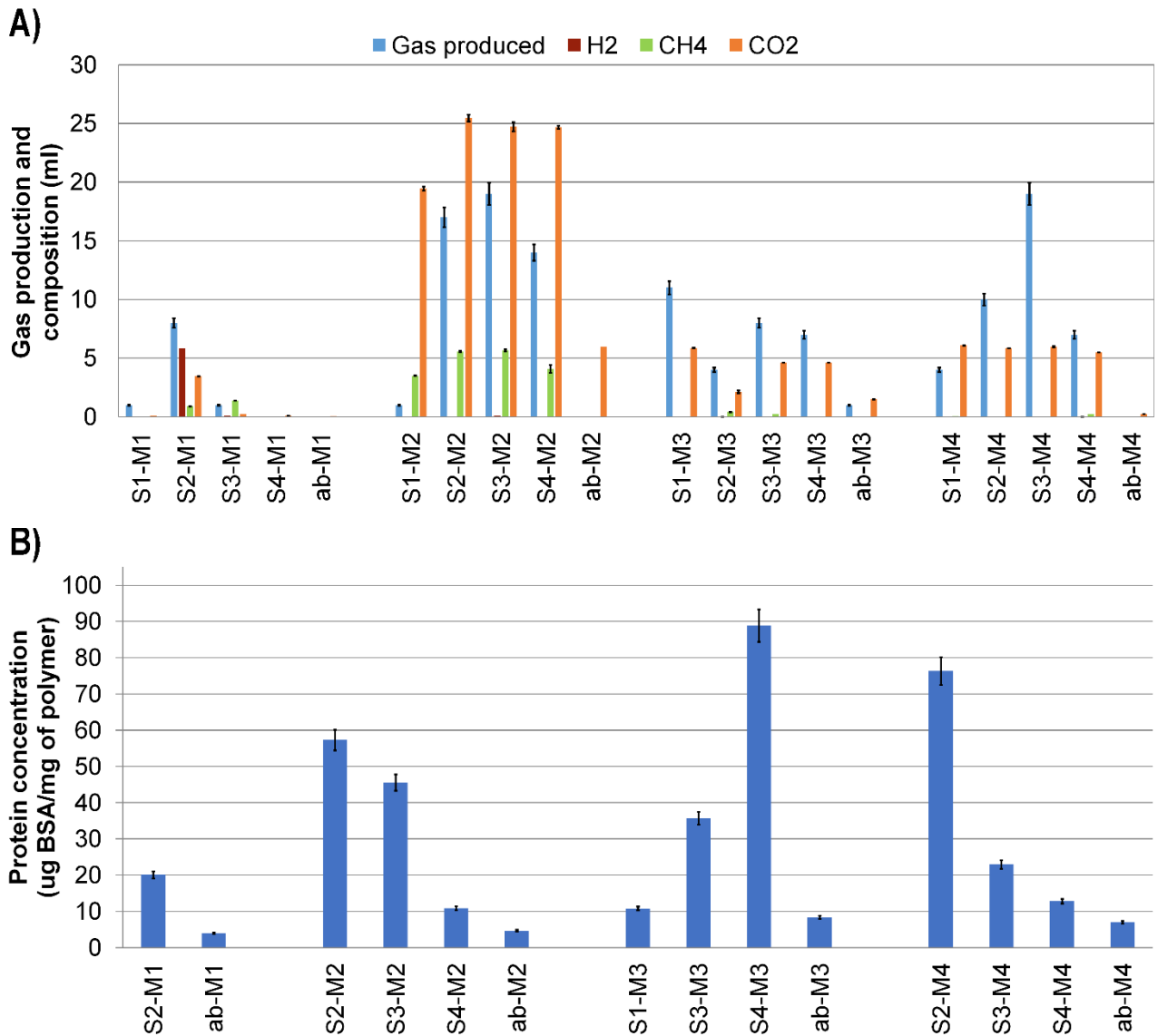
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636 FEMS Microbiol. Ecol. 59, 706-717. DOI: 10.1111/j.1574-6941.2006.00248.x
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638

639 **Figure 1.** Evaluation of microbial growth and biofilm formation after 7 months incubation.

640 Volume and composition of gases produced by the sixteen different enriched marine consortia (the

641 combination of 4 native marine/litter communities, i.e. S1 to S4, grown in the presence of 4

642 different media, i.e. M1 to M4) (a); Concentration of proteins adhered on surfaces of PVC films

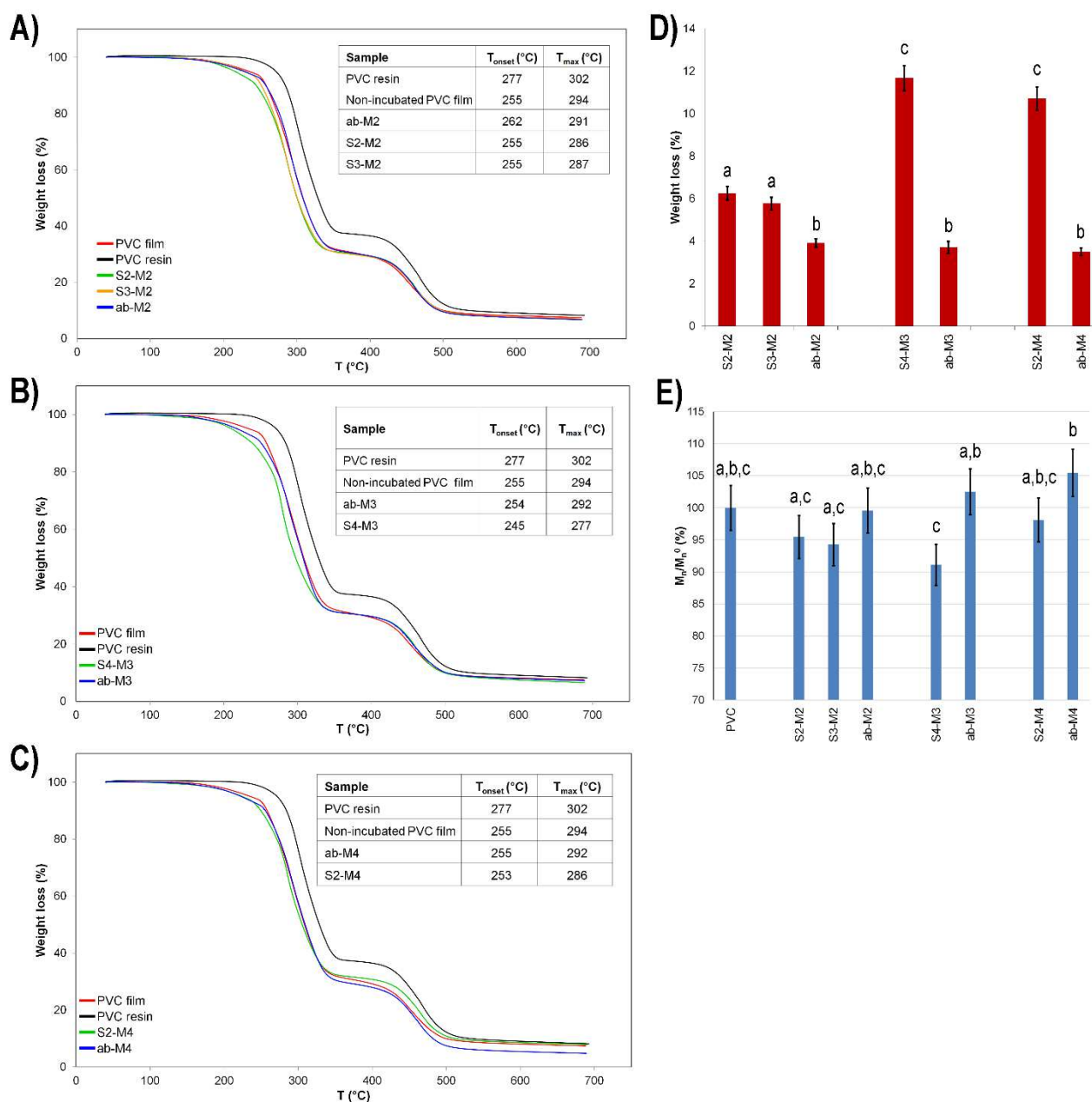
643 incubated under abiotic conditions (ab-M1 to ab-M4) or with the ten different enriched marine

644 consortia showing highest microbial growth by gas analysis (b). Bars indicate standard deviation.

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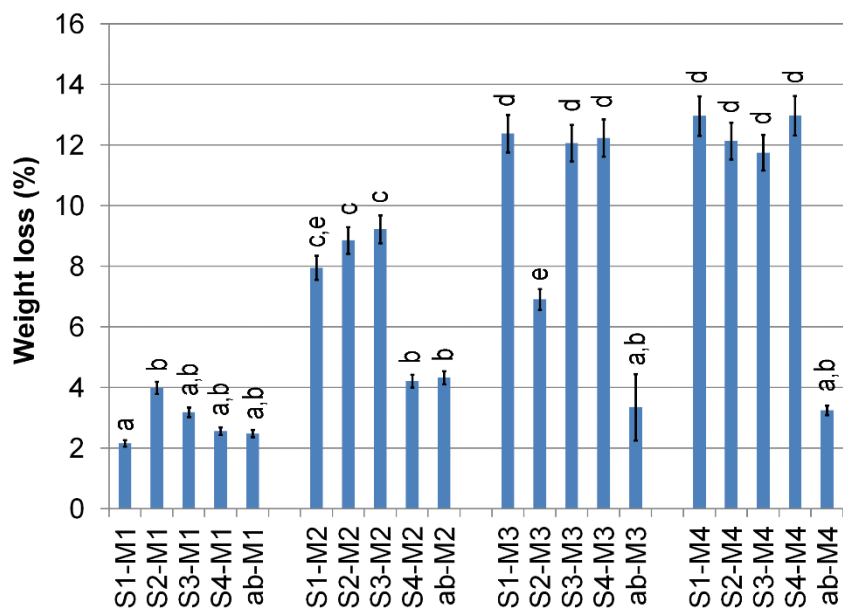


648

649 **Figure 2.** Chemical-physical analyses on PVC films withdrawn at month 7. TGA curves of PVC
650 films incubated with the four different consortia enriched in M2 (A), M3 (B) and M4 (C) media or
651 in the four corresponding abiotic controls. Gravimetric weight loss percentage of PVC films
652 incubated with the four different consortia or in the four corresponding abiotic controls (D)
653 (ANOVA: $p < 0.0001$; $F = 249.9$; $df = 6$). Number average molecular weight (Mn) of selected PVC
654 films (ANOVA: $p = 0.0025$; $F = 5.42$; $df = 7$) (E). Mn (%) is defined as $(Mn/Mn_0) \cdot 100$, where Mn
655 is the molecular weight at sampling time t and Mn₀ is the molecular weight of the non-incubated
656 PVC film. Bars indicate standard deviation. Letters a, b, c show results of post-hoc analyses for film

657 gravimetric or molecular weight losses ($p < 0.05$), different letters indicate a significant statistical
658 difference in gravimetric or molecular weight reduction values.

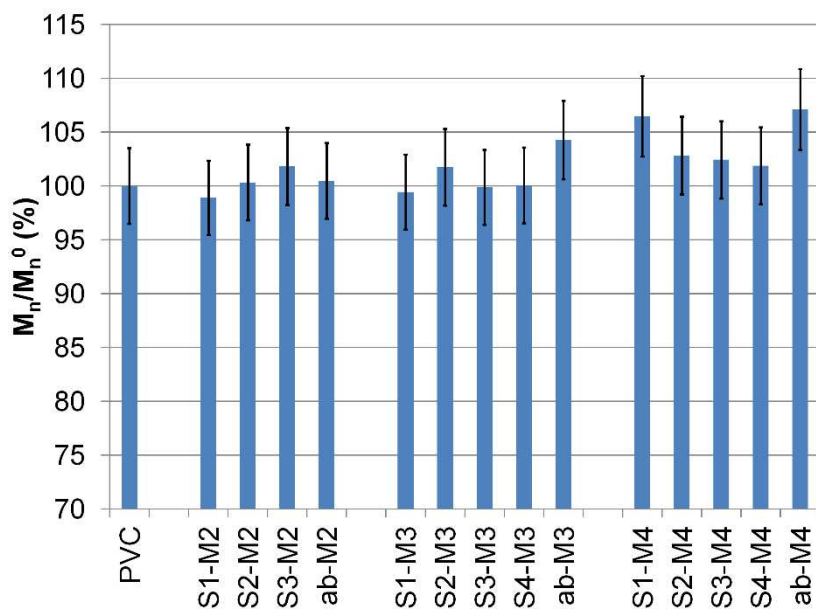
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661 **Figure 3.** Gravimetric measurements at month 24. Weight loss (%) of PVC films incubated with
662 the sixteen enriched consortia and the corresponding four abiotic controls. Bars indicate standard
663 deviation (ANOVA: $p < 0.0001$; $F = 222.3$; $df = 19$). Letters a, b, c, d, e show results of post-hoc
664 analyses for film gravimetric or molecular weight losses ($p < 0.05$), different letters indicate a
665 significant statistical difference in gravimetric weight reduction values.

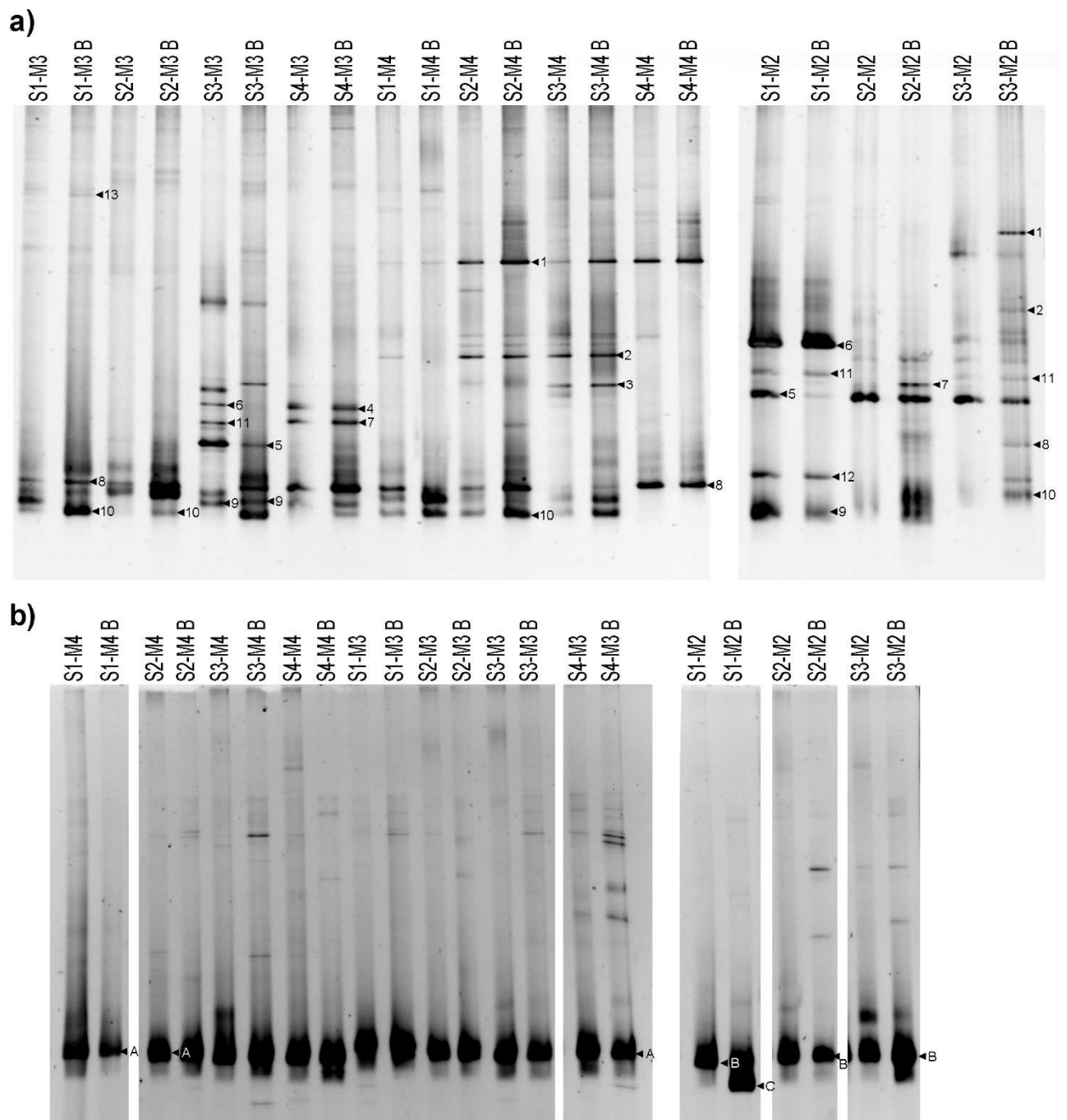
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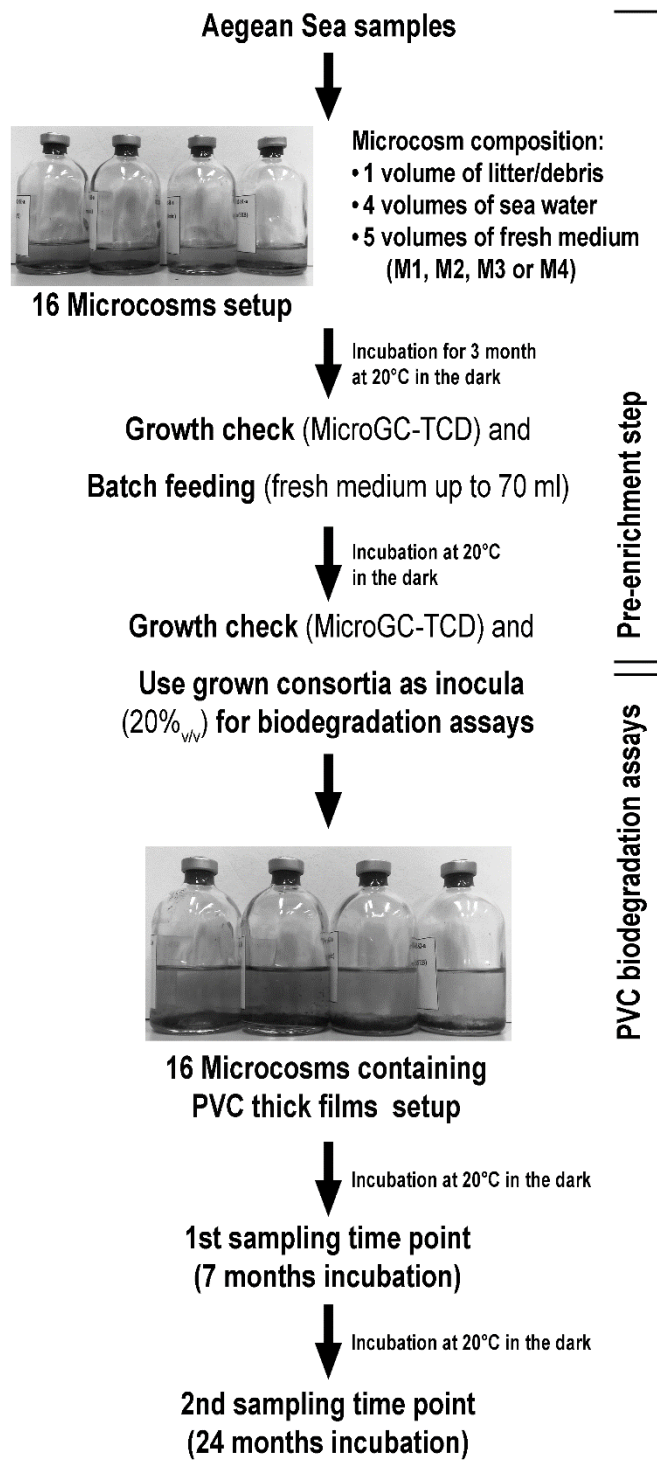
668 **Figure 4.** PVC mean molecular weight at month 24. GPC results for PVC films incubated with
 669 eleven different consortia enriched in M2, M3 and M4 media and their corresponding abiotic
 670 controls (ab-M1; ab-M2; ab-M3 and ab-M4) (ANOVA: $p = 0.19$; $F = 1.44$; $df = 14$).

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673 **Figure 5.** Bacterial (a) and archaeal (b) diversities of the 24 months PVC film-enriched anaerobic
 674 communities. DGGE profiles of 16S rRNA genes amplified from planktonic and biofilm microbial
 675 communities from the eleven different consortia showing high weight losses compared to the
 676 corresponding abiotic controls. Names over the lanes refer to the ID of the consortium. The letter B
 677 after consortia names stands for “biofilm community”. The identity of sequences of bands marked
 678 with arrows is given in Table 2 according to the band ID (1 to 13 and A to C).



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680 **Figure S1.** Experimental design. Schematic presentation of the experimental sequence for
 681 assessment of PVC film biodegradation.

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685 **Supplementary material – S1**

686 Supplementary material S1 contains the composition of the four media used to enrich the different marine
687 anaerobic consortia and used to perform PVC biodegradation study.

688 *Medium M1* was composed of (g/l): sodium formate, 2; Na₂CO₃, 0.1; (NH₄)₂SO₄, 0.5; mineral solution (per
689 litre: KH₂PO₄, 18 g; NaCl, 18 g; CaCl₂•2H₂O, 0.53 g; MgCl₂•6H₂O, 0.4 g; MnCl₂•4H₂O, 0.2 g; CoCl₂•6H₂O,
690 0.02 g), 50 ml; acetic acid, 1.53 ml; vitamin solution (per litre: thiamine-HCl, 20 mg; Ca-D-pantothenate, 20
691 mg; nicotinamide, 20 mg; riboflavin, 20 mg; pyridoxine-HCl, 20 mg; biotin, 0.5 mg; folic acid, 0.25 mg;
692 vitamin B₁₂, 0.2 mg), 5 ml; hemin solution (10 mg in 100 ml of 50% ethanolic), 5 ml; cysteine-sulphide solution
693 (L-cysteine-HCl•H₂O, 2.5 g/l; Na₂S•9H₂O, 2.5 g/l; pH 11), 5 ml; pH 6.7 (Cacciari et al. 1993).

694 *Medium M2* was composed of (g/l): glucose, 2; yeast extract, 1 g; tryptone, 1 g; beef extract, 0.5 g; K₂HPO₄,
695 1.5 g; MgCl₂•6H₂O, 0.21 g; FeSO₄•7H₂O, 0.1 g; trace element solution (per litre: MnSO₄•7H₂O, 0.01 g;
696 ZnSO₄•7H₂O, 0.05 g; H₃BO₃, 0.01 g; CaCl₂•2H₂O, 0.01 g; Na₂MoO₄, 0.01 g; CoCl₂•6H₂O, 0.2 g), 10 ml;
697 vitamin solution (per litre: L-ascorbic acid, 0.025 g; citric acid, 0.02 g; pyridoxine-HCl, 0.05 g; para-
698 aminobenzoic acid, 0.01 g; D-biotin, 0.01 g; vitamin B₁, 0.02 g; riboflavine, 0.025 g) 1 ml; 5 %_{w/v} L-cysteine
699 solution, 10 ml; pH 7.2-7.5.

700 *Medium M3* was composed of (g/l): glucose, 2; KH₂PO₄, 0.5; NH₄Cl, 0.3; MgSO₄•7H₂O, 0.5; CaCl₂•2H₂O,
701 0.1; NaNO₃, 3; NaHCO₃ solution, 30 ml; vitamins solution, 1 ml; EDTA-chelated mixture of trace elements,
702 1 ml; selenite-tungstate solution, 1 ml; pH 7.2. All solutions composition were from Widdel and Bak (1992).

703 *Medium M4* was composed per litre of distilled water: sodium lactate, 4.5 g; Na₂SO₄, 6 g; KH₂PO₄, 0.2 g;
704 NH₄Cl, 0.25 g; MgCl₂•6H₂O, 0.4 g; KCl, 0.5 g; CaCl₂•2H₂O, 0.15 g; DSMZ SL-10 trace elements solution, 1
705 ml; selenite-tungstate solution (Widdel and Bak, 1992), 1 ml; mixed vitamin solution (per 100 ml of 10 mM
706 sodium phosphate pH 7.1: p-aminobenzoic acid, 4 mg; biotin, 1 mg; nicotinic acid, 10 mg; D,L-Ca
707 pantothenate, 5 mg; pyridoxine-HCl, 15 mg; thiamine-HCl, 10 mg), 1 ml; 0.005%_{w/v} vitamin B12 solution, 1
708 ml; 84 mg/l CaHCO₃ solution, 30 ml; 4%_{w/v} sulfide solution, 7.5 ml; pH 7.0-7.5.

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711 **Tables**

712 **Table 1. TGA at month 24.** Results of TGA of PVC resin, PVC film (non-incubated) and PVC films incubated under abiotic conditions (ab-M2
713 to ab-M4) or with the consortia enriched in three media that showed lower thermal stability compared to abiotic controls.

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Sample	T _{onset} (°C)	T _{max} (°C)
PVC resin	277	302
PVC film	255	294
ab-M2	261	290
S2-M2	260	284
S3-M2	259	283
ab-M3	260	289
S4-M3	263	285
ab-M4	260	289
S1-M4	263	283
S2-M4	262	285
S4-M4	261	284

Table 2. Identification of microorganisms characterizing the different PVC film associated communities according to DGGE profiles.

Band ID	Closest cultured relative (BLAST)	Accession No.	Identity (nt%)	Putative classification	No. positives ^a
<i>Bacteria</i>					
1	<i>Erysipelothrix</i> sp. (KX156777)	MH520751	95	<i>Erysipelotrichaceae</i>	5/11
2	Uncultured <i>CFB</i> group bacterium (FJ024711) *	MH520752	98	<i>CFB</i> group	3/11
3	<i>Bacteroidales</i> bacterium CF (CP006772)	MH520753	93	<i>Bacteroidetes</i>	2/11
4	<i>Psychromonas</i> sp. (NR116830)	MH520754	99	<i>Psychromonas</i>	1/11
5	<i>Cupriavidus</i> sp. (MG948149)	MH520755	100	<i>Cupriavidus</i>	4/11
6	<i>Pleomorphochaeta</i> sp. (NR134177)	MH520756	98	<i>Pleomorphochaeta</i>	4/11
7	<i>Sporobacter</i> sp. (KT183425)	MH520757	99	<i>Ruminococcaceae</i>	3/11
8	<i>Clostridium</i> sp. a-nd (FN397991)	MH520758	98	<i>Clostridiaceae</i>	9/11
9	<i>Dethiosulfovibrio</i> sp. (NR029034)	MH520759	100	<i>Dethiosulfovibrio</i>	3/11
10	<i>Acetobacterium</i> sp. (NR074548)	MH520760	98	<i>Acetobacterium</i>	8/11
11	<i>Cohaesibacter</i> sp. (KT324976)	MH520761	99	<i>Cohaesibacter</i>	2/11
12	<i>Desulfovibrio</i> sp. (KU892724)	MH520762	99	<i>Desulfovibrio</i>	1/11
13	<i>Fusibacter</i> sp. (KJ420408)	MH520763	96	<i>Fusibacter</i>	1/11
<i>Archaea</i>					
A	<i>Methanosaeta pelagica</i> (NR113571)	MH520764	99	<i>Methanosaeta</i>	8/11
B	<i>Methanococcoides methylutens</i> (CP009518)	MH520765	99	<i>Methanococcoides</i>	3/11
C	<i>Methanogenium</i> sp. (NR104730)	MH520766	99	<i>Methanogenium</i>	1/11

724

725 ^a Number of samples positive for the presence of the specific band in DGGE analysis compared to total number of PVC plastic associated biofilm
726 communities analyzed.

727 * The sequence related to band #2 demonstrated a very low homology with cultivable bacteria, it was analyzed including the
728 uncultured/environmental sample sequences present in nucleotide databases.

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