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Biodegradation of polyvinyl chloride plastic films by enriched anaerobic marine consortia

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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±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.

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1. Introduction

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

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

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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 as source of microbial communitites. 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 ($N_2:CO_2=80:20$, for the

119 M2 medium) and incubated at 20°C. Microbial growth was evaluated after 3 months incubation

based on gas composition analysis of the headspace of each microcosm (H_2, CO_2, CH_4) by gas

chromatography (Agilent 3000 MicroGC, Agilent technologies, Santa Clara, CA, USA) equipped

with thermal conductivity detector (TCD). The analytical conditions were as follows: injector

temperature 90 °C; column temperature 60 °C; sampling time 20 s; injection time 50 ms; column

pressure 25 psi; run time 44 s; carrier gas N_2. When microbial growth was detected, fresh medium

was added to a final volume of 70 ml and microcosms were incubated under the same conditions

until a significant growth was detected, before using as inocula in the biodegradation experiment.

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

2.2. PVC film

PVC film containing a total of 30%$_{w/w}$ of additives was provided by an Italian plastic producing

company. In order to better monitor the gravimetric weight loss, thick PVC plastic films (300 μm),

obtained after pressing several PVC thin layers (20 μm each) at 17.44 bar and 160°C for 4-5 min,

were used. Thick films were then cut into small pieces (1.5 cm × 1.5 cm and 1.5 cm × 3.2 cm),

degreased and sterilized by 30 min immersion in 70%$_{v/v}$ ethanol solution and washed with sterile

distilled water before use. The films used for the determination of gravimetric weight loss were

dried under vacuum to constant weight before being used as substrate for microbial growth.

2.3. PVC biodegradation assays

The sixteen microbial communities from the pre-enrichment step were used as inocula for PVC

biodegradation experiments (Figure S1). Specifically, sixteen microcosms were setup in 100 ml

serum bottles with 50 ml of the corresponding medium containing 10 g/l of PVC films as major

carbon and energy source and inoculated with 20%$_{v/v}$ of pre-enriched cultures. Inoculated

microcosms and abiotic controls were incubated under static conditions at 20°C. Samples of plastic

144 films and culture broth were withdrown after 7 and 24 months incubation and subjected to the

145 following analyses in order to detect putative microbial growth and PVC films biodegradation.

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 (H_2, CO_2, CH_4) 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.

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 Weight loss (%) = (Initial weight-Final weight)/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

standards of known molecular mass. A sample concentration of 3 mg/ml was applied. Non

incubated PVC film and PVC films incubated under abiotic conditions were used as references.

2.3.3. Statistical analyses

Results of gravimetric weight losses and GPC analyses were statistically evaluated using one-way

ANOVA. Post-hoc Tukey test was applied to determine whether gravimetric or molecular weight

changes of PVC films showed significantly different extents. Statistically significant results were

depicted by *p-values* < 0.05. All the analyses were performed using GraphPad Prism version 8.0.0

for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

2.4. Composition of planktonic and biofilm communities

After 24 months incubation, total DNA was extracted using a Powersoil® DNA isolation kit (MOBIO,

Carlsbad, CA, USA) from planktonic (from 2 ml of culture broth) and biofilm (PVC adhered

microbial cells) microbial communities. Bacterial partial 16S rRNA gene was amplified using 357F-

GC and 907R primers and PCR conditions reported by Polo *et al.* (2010) except for $MgCl_2$ and dNTP

mix concentration of 1.8-2 mM and 0.2 μM, respectively and annealing temperature of 55°C.

Archaeal PCRs were performed using Arch344F-GC and Arch918R (Giovannoni *et al.*, 1988; Stahl

and Amann, 1991) primer pair. The archaeal thermal protocol was a modification of the bacterial

protocol by elongating each step of 15 sec. DGGE was according to Polo *et al.* (2010), using

denaturant gradients of 40–60% and 30–70% for bacterial and archaeal communities, respectively.

Principal bands were excised, eluted in 50 μl milliQ water (30°C for 3 h), amplified and identified by

sequencing (Macrogen Inc., Republic of Korea). The sequences were checked for chimeras using

DECIPHER software (Wright *et al.*, 2012), analyzed using the BLASTN software and submitted to

the Genbank database (accession numbers MH520751 to MH520766).

9

3. Results and discussion

3.1. PVC films biodegradability after 7 months

3.1.1. Microbial growth monitoring

Before performing the biodegradation assays, a pre-enrichment step (growth in the absence of PVC film) was performed (please refer to the scheme of the experimental design in supplementary Figure S1) and microbial growth was confirmed by headspace gas analysis. The pre-enriched consortia were used as inocula in the biodegradation assays. Microbial growth in the presence of PVC films as major carbon and energy source was monitored by measuring the volume and composition of the gas produced in the headspace of each consortium. After 7 months incubation, significant gas volumes were measured in 10 microcosms (Figure 1A) reaching a maximum of 19.0 ± 1.0 ml, while no gas production was detected in the four abiotic controls. Headspace gas analysis showed that CO_2 was produced in all the microcosms while hydrogen was recorded only in the case of S2-M1 consortium (5.8 ± 0.0 ml) (Figure 1A). Methane (max volume = 5.7 ± 0.1 ml) was produced by the four consortia enriched in M2 medium, while very low/no methane production was recorded in microcosms enriched in M1, M3 and M4 media. These results showed that growth of the native marine microbial communities occurred in the presence of PVC plastic film as major C and energy source. Plasticizers probably sustained the observed growth, in line with previous studies carried out with PVC plastic under sulfate reducing conditions with bacteria enriched from landfill (Tsuchida *et al.*, 2011).

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

3.1.2. Evaluation of microbial colonization of PVC film surface.

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

10

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±SD) of 57.3±2.9, 45.6±2.3, 88.9±4.4 and 76.3±3.8 µg

221 of BSA equivalent/mg of PVC film, respectively. Microbial colonization of PVC films was reported

222 under aerobic conditions. Specifically, unpretreated 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).

3.1.3. Evaluation of PVC biodegradation

Biodegradation activity was evaluated on film samples withdrawn from the four (S2-M2, S3-M2,

S4-M3 and S2-M4) selected microcosms. Gravimetric measurements revealed a statistically higher

decrease in their weights ($p < 0.05$; Figure 2 A-D) compared to those recovered from the

corresponding abiotic controls. Specifically, weight reductions (mean±SD%) of 11.67±0.58%,

10.71±0.54%, 6.26±0.31% and 5.77±0.29% were recorded for the films incubated in S4-M3, S2-

M4, S2-M2 and S3-M2 microcosms, respectively. Among the biological effect of the four selected

consortia, the highest gravimetric weight decrease was recorded by films incubated with S4-M3 and

S2-M4 consortia, which showed the same statistical film weight reduction followed by the action of

S2-M2 and S3-M2 consortia ($p < 0.05$; Figure 2 D). The corresponding abiotic controls incubated in

the growth media M3, M4 and M2 showed statistically equal weight losses of 3.71±0.28%,

3.91±0.2% and 3.51±0.18% respectively ($p < 0.05$; Figure 2 A-D) due to additives leaching from

the PVC films (Kastner *et al.*, 2012; Suhrhoff and Scholz-Bottcher, 2016; Wang *et al.*, 2016).

These evidences are the first proof of plastic biodegradation; indeed, the reduction of gravimetric

weight, which is a direct evidence of the loss of material, is widely used in biodegradation tests

(Shah *et al.* 2008; Wang *et al.*, 2016). Thus some components of the PVC films were degraded by

the enriched microbial communities. Since the films used in this study had about 30%$_{w/w}$ of

additives, further analyses were performed in order to elucidate if the biodegradation regarded the

sole additives or also the PVC polymer.

TGA showed a lower thermal stability of the films recovered from the four active microcosms

(Figure 2A-C) compared to those incubated under abiotic conditions. Specifically, a T_{max} ranging

from 287°C to 277°C was recorded for the former compared to a T_{max} of 292°C for those of abiotic

controls or 294°C for the non incubated PVC film. Considering that the presence of additives

(citrates, polyadipates, epoxidized soybean oil, Zn etc; data supplied by the production company) is

lowering the thermal stability of PVC resins once in PVC plastic film, the (bio)degradation of the

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±SD) from

272 100% to 91.1±3.2%, 94.3±3.3%, 95.5±3.3%, and 98.1±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 biodegdataion 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±3.7% (mean±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

13

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±SD)

297 of the PVC films by up to 12.97±0.65% ($p < 0.05$; showed by d letter in Figure 3). A statistically

298 lower weight loss of up to 9.23±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±0.21%, statistically comparable to that recorded under abiotic conditions

301 (4.32±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 Mn (%) 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

14

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*

15

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) *Methanococcoides 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 *Dehalococcoides*-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 H_2 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 synthetizing 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

17

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 archaeael 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

18

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.

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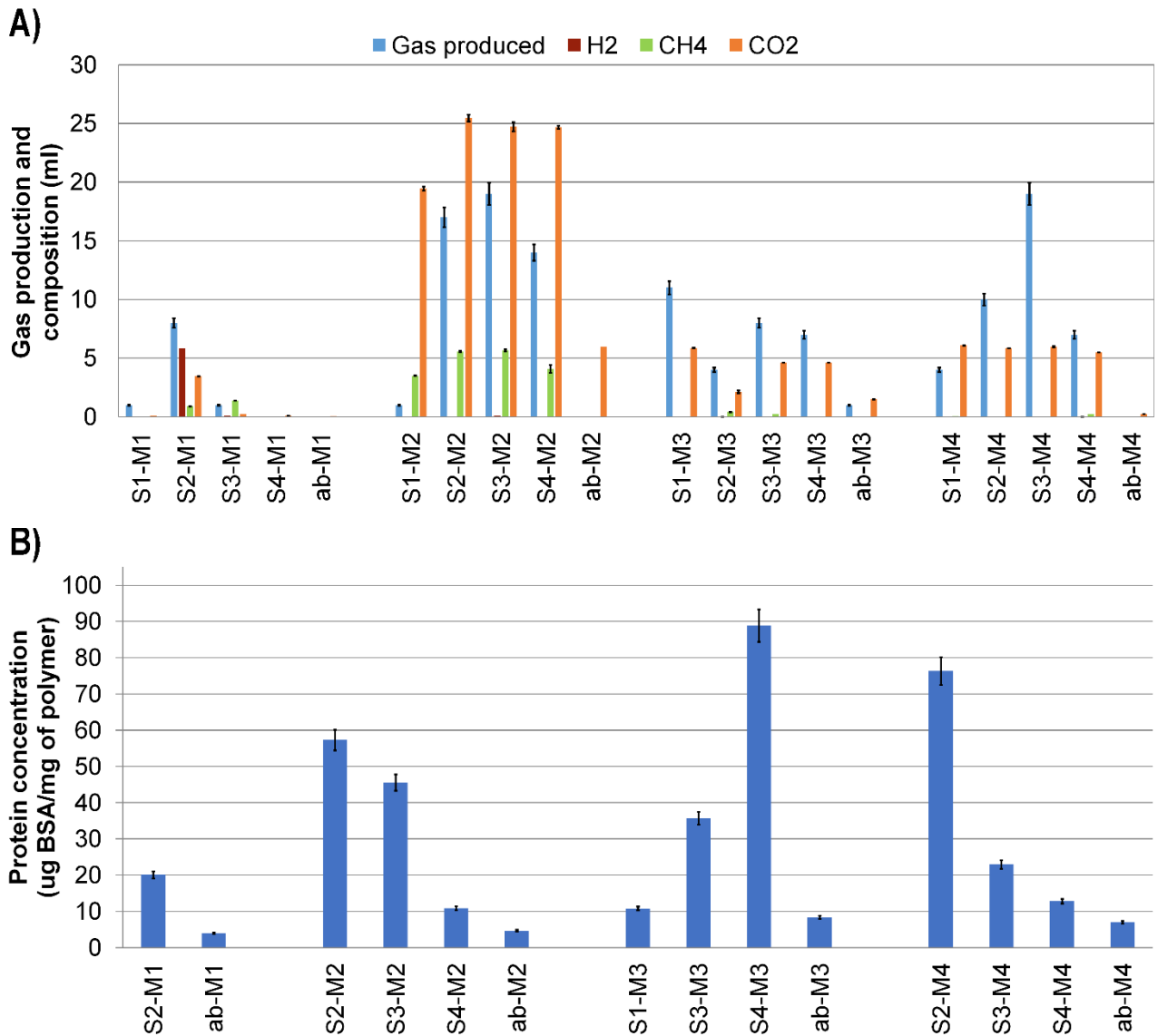
Figure 1. Evaluation of microbial growth and biofilm formation after 7 months incubation. Volume and composition of gases produced by the sixteen different enriched marine consortia (the combination of 4 native marine/litter communities, i.e. S1 to S4, grown in the presence of 4 different media, i.e. M1 to M4) (a)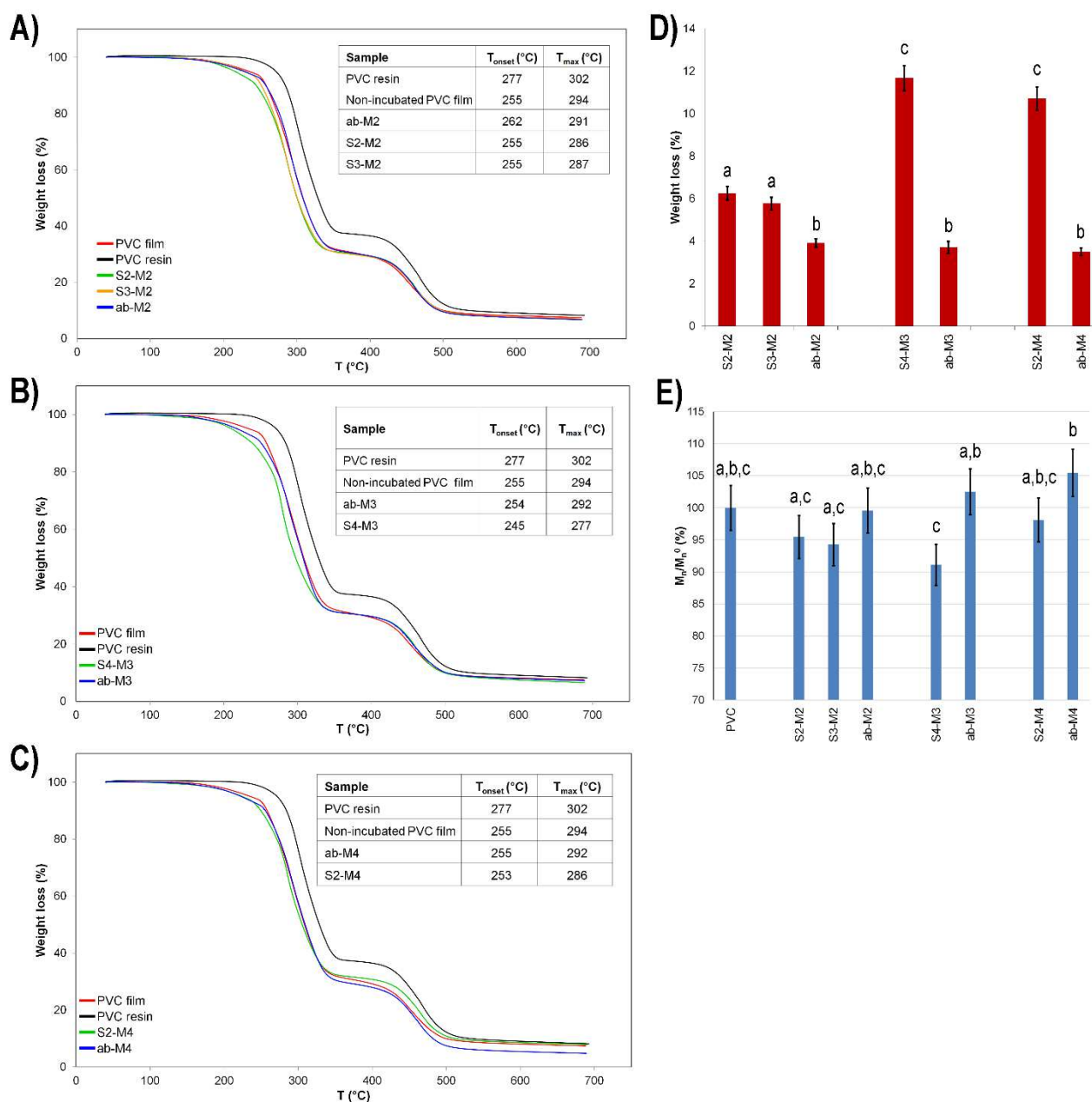; Concentration of proteins adhered on surfaces of PVC films incubated under abiotic conditions (ab-M1 to ab-M4) or with the ten different enriched marine consortia showing highest microbial growth by gas analysis (b). Bars indicate standard deviation.

Figure 2. Chemical-physical analyses on PVC films withdrawn at month 7. TGA curves of PVC

films incubated with the four different consortia enriched in M2 (A), M3 (B) and M4 (C) media or

in the four corresponding abiotic controls. Gravimetric weight loss percentage of PVC films

incubated with the four different consortia or in the four corresponding abiotic controls (D)

(ANOVA: $p < 0.0001$; $F = 249.9$; $df = 6$). Number average molecular weight (Mn) of selected PVC

films (ANOVA: $p = 0.0025$; $F = 5.42$; $df = 7$) (E). Mn (%) is defined as (Mn/Mn0)*100, where Mn

is the molecular weight at sampling time t and Mn0 is the molecular weight of the non-incubated

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.

659
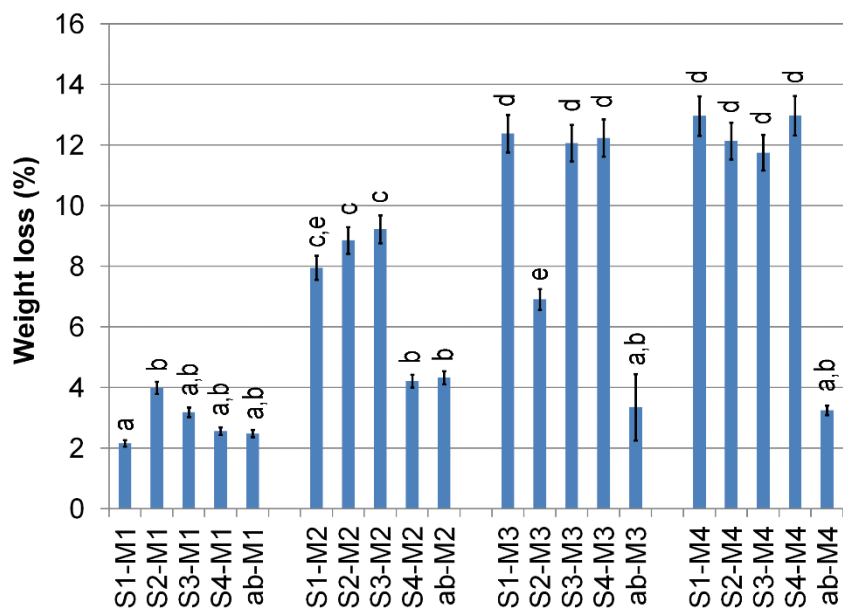
660

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

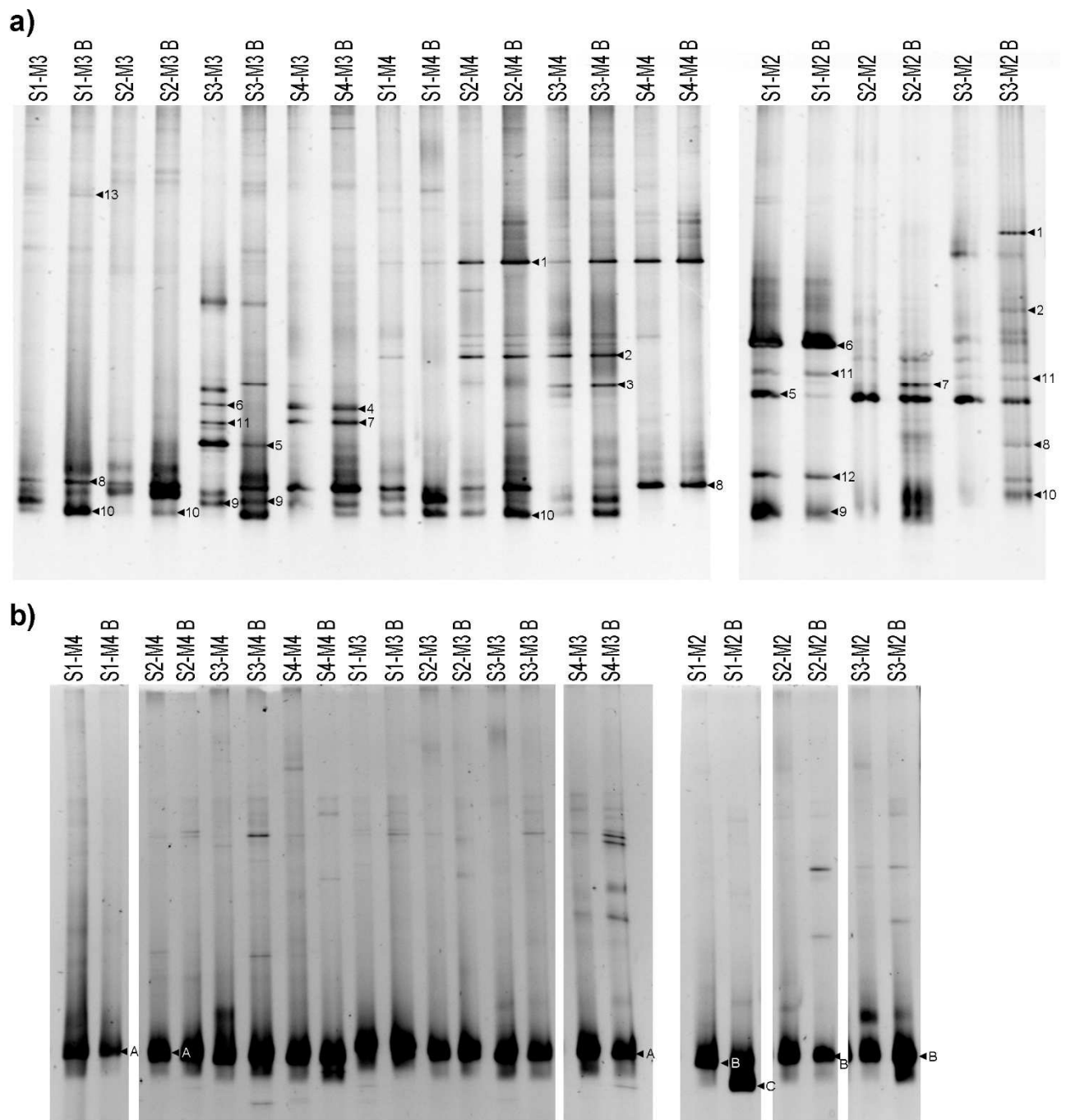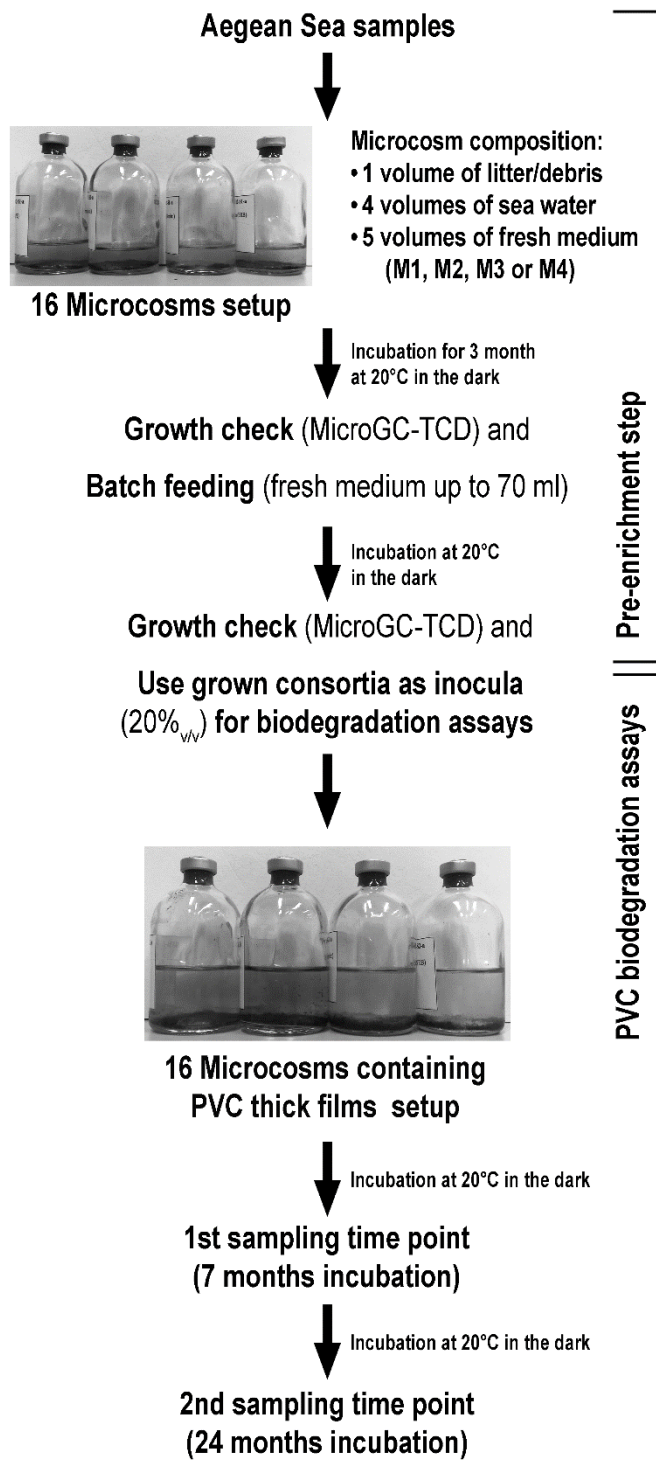665   significant statistical difference in gravimetric weight reduction values.

666

667
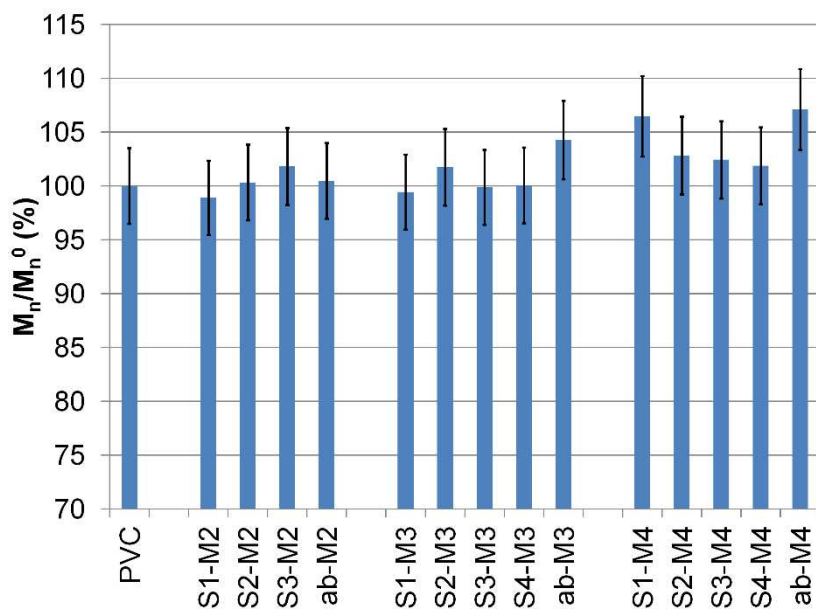
Figure 4. PVC mean molecular weight at month 24. GPC results for PVC films incubated with

eleven different consortia enriched in M2, M3 and M4 media and their corresponding abiotic

controls (ab-M1; ab-M2; ab-M3 and ab-M4) (ANOVA: p = 0.19; F = 1.44; df = 14).

671

Figure 5. Bacterial (a) and archaeal (b) diversities of the 24 months PVC film-enriched anaerobic

communities. DGGE profiles of 16S rRNA genes amplified from planktonic and biofilm microbial

communities from the eleven different consortia showing high weight losses compared to the

corresponding abiotic controls. Names over the lanes refer to the ID of the consortium. The letter B

after consortia names stands for "biofilm community". The identity of sequences of bands marked

with arrows is given in Table 2 according to the band ID (1 to 13 and A to C).

Aegean Sea samples

Microcosm composition:
• 1 volume of litter/debris
• 4 volumes of sea water
• 5 volumes of fresh medium
  (M1, M2, M3 or M4)

16 Microcosms setup

Incubation for 3 month
at 20°C in the dark

Growth check (MicroGC-TCD) and

Batch feeding (fresh medium up to 70 ml)

Incubation at 20°C
in the dark

Growth check (MicroGC-TCD) and

**Use grown consortia as inocula
(20%$_{v/v}$) for biodegradation assays**

Pre-enrichment step

**16 Microcosms containing
PVC thick films setup**

PVC biodegradation assays

Incubation at 20°C in the dark

**1st sampling time point
(7 months incubation)**

Incubation at 20°C in the dark

**2nd sampling time point
(24 months incubation)**

679

680   **Figure S1.** Experimental design. Schematic presentation of the experimental sequence for

681   assessment of PVC film biodegradation.

682

683

684

Supplementary material – S1

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

Medium M1 was composed of (g/l): sodium formate, 2; Na_2CO_3, 0.1; $(NH_4)_2SO_4$, 0.5; mineral solution (per litre: KH_2PO_4, 18 g; NaCl, 18 g; $CaCl_2•2H_2O$, 0.53 g; $MgCl_2•6H_2O$, 0.4 g; $MnCl_2•4H_2O$, 0.2 g; $CoCl_2•6H_2O$, 0.02 g), 50 ml; acetic acid, 1.53 ml; vitamin solution (per litre: thiamine-HCl, 20 mg; Ca-D-pantothenate, 20 mg; nicotinamide, 20 mg; riboflavin, 20 mg; pyridoxine-HCl, 20 mg; biotin, 0.5 mg; folic acid, 0.25 mg; vitamin B_{12}, 0.2 mg), 5 ml; hemin solution (10 mg in 100 ml of 50% ethanolic), 5 ml; cysteine-sulphide solution (L-cysteine-HCl•H_2O, 2.5 g/l; $Na_2S•9H_2O$, 2.5 g/l; pH 11), 5 ml; pH 6.7 (Cacciari et al. 1993).

Medium M2 was composed of (g/l): glucose, 2; yeast extract, 1 g; tryptone, 1 g; beef extract, 0.5 g; K_2HPO_4, 1.5 g; $MgCl_2•6H_2O$, 0.21 g; $FeSO_4•7H_2O$, 0.1 g; trace element solution (per litre: $MnSO_4•7H_2O$, 0.01 g; $ZnSO_4•7H_2O$, 0.05 g; H_3BO_3, 0.01 g; $CaCl_2•2H_2O$, 0.01 g; Na_2MoO_4, 0.01 g; $CoCl_2•6H_2O$, 0.2 g), 10 ml; vitamin solution (per litre: L-ascorbic acid, 0.025 g; citric acid, 0.02 g; piridoxine-HCl, 0.05 g; para-aminobenzoic acid, 0.01 g; D-biotin, 0.01 g; vitamin B_1, 0.02 g; riboflavine, 0.025 g) 1 ml; 5 %$_{w/v}$ L-cysteine solution, 10 ml; pH 7.2-7.5.

Medium M3 was composed of (g/l): glucose, 2; KH_2PO_4, 0.5; NH_4Cl, 0.3; $MgSO_4•7H_2O$, 0.5; $CaCl_2•2H_2O$, 0.1; $NaNO_3$, 3; $NaHCO_3$ solution, 30 ml; vitamins solution, 1 ml; EDTA-chelated mixture of trace elements, 1 ml; selenite-tungstate solution, 1 ml; pH 7.2. All solutions composition were from Widdel and Bak (1992).

Medium M4 was composed per litre of distilled water: sodium lactate, 4.5 g; Na_2SO_4, 6 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; $MgCl_2•6H_2O$, 0.4 g; KCl, 0.5 g; $CaCl_2•2H_2O$, 0.15 g; DSMZ SL-10 trace elements solution, 1 ml; selenite-tungstate solution (Widdel and Bak, 1992), 1 ml; mixed vitamin solution (per 100 ml of 10 mM sodium phosphate pH 7.1: p-aminobenzoic acid, 4 mg; biotin, 1 mg; nicotinic acid, 10 mg; D,L-Ca pantothenate, 5 mg; pyridoxine-HCl, 15 mg; thiamine-HCl, 10 mg), 1 ml; 0.005%$_{w/v}$ vitamin B12 solution, 1 ml; 84 mg/l $CaHCO_3$ solution, 30 ml; 4%$_{w/v}$ sulfide solution, 7.5 ml; pH 7.0-7.5.

Tables

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 to ab-M4) or with the consortia enriched in three media that showed lower thermal stability compared to abiotic controls.

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

723 **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]
Bacteria					
1	*Erysipelothrix* sp. (KX156777)	MH520751	95	*Erysipelotrichaceae*	5/11
2	Uncultured *CFB group* bacterium (FJ024711) *	MH520752	98	*CFB group*	3/11
3	*Bacteroidales bacterium* CF (CP006772)	MH520753	93	*Bacteroidetes*	2/11
4	*Psychromonas* sp. (NR116830)	MH520754	99	*Psychromonas*	1/11
5	*Cupriavidus* sp. (MG948149)	MH520755	100	*Cupriavidus*	4/11
6	*Pleomorphochaeta* sp. (NR134177)	MH520756	98	*Pleomorphochaeta*	4/11
7	*Sporobacter* sp. (KT183425)	MH520757	99	*Ruminococcaceae*	3/11
8	*Clostridium* sp. a-nd (FN397991)	MH520758	98	*Clostridiaceae*	9/11
9	*Dethiosulfovibrio* sp. (NR029034)	MH520759	100	*Dethiosulfovibrio*	3/11
10	*Acetobacterium* sp. (NR074548)	MH520760	98	*Acetobacterium*	8/11
11	*Cohaesibacter* sp. (KT324976)	MH520761	99	*Cohaesibacter*	2/11
12	*Desulfovibrio* sp. (KU892724)	MH520762	99	*Desulfovibrio*	1/11
13	*Fusibacter* sp. (KJ420408)	MH520763	96	*Fusibacter*	1/11
Archaea					
A	*Methanosaeta pelagica* (NR113571)	MH520764	99	*Methanosaeta*	8/11
B	*Methanococcoides methylutens* (CP009518)	MH520765	99	*Methanococcoides*	3/11
C	*Methanogenium* sp. (NR104730)	MH520766	99	*Methanogenium*	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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730